Growth characteristics of *Campylobacter sputorum* subspecies *mucosalis* in cell cultures.

MALLESHAPPA RAJASEKHAR

A thesis submitted for the degree of Doctor of Philosophy.

University of Edinburgh.

1981.

DECLARATION

I certify that the work presented in this thesis is my own.

Malleshappa Rajasekhar. 23.7.81

Acknowledgements

I would like to thank all the people who have assisted me during the course of this thesis, and especially my two supervisors Drs. G.H.K. Lawson and G. Fraser for their guidance, advice and reading of the manuscript. My thanks are also due to Dr R.W. Bingham, for his help and assistance with electron microscopic studies. I am particularly pleased to acknowledge the excellent technical ability and unstinted efforts of Mr B. Kelly in the preparation of specimens for electron microscopy. I have received much valuable assistance from Mrs S. Shaw, Miss P. Wooding, Mr I. Bennet and Mr D. Veitch, as well as from the staff of the media and glassware preparation room. I am indebted to Mr B. Munro, Mr K. Thompson and Mr R.C. James for the excellent production of photographs and to the staff of the electron microscope unit for their help during my study. The typing of the manuscript has been speedily performed by Miss A. Laird.

This work is made possible by a Commonwealth academic staff scholarship sponsored by the University of Agricultural Sciences, Bangalore, University Grants Commissions of India and U.K.

Finally, I wish to record my appreciation of the patience and understanding shown by my wife and family in particular, my mother.

CONTENTS

CONTENTS.

SUMMARY

SUMMARY

In this study, the growth characteristics of Campylobacter sputorum ss mucosalis were investigated in twelve different types of cell cultures: primary cultures of porcine kidney (PPK), chicken embryo fibroblasts (CEF) and cell lines of porcine kidney (PK), bovine kidney (BK), canine kidney (DK), monkey kidney (Vero and $LLCMK_2$), baby hamster kidney (BHK-21), human cervical carcinoma (HeLa) cells and lines of porcine (PK_{pi}), bovine (BK_{pi}) and ovine (OK_{pi}) kidney cells persistently infected with Newcastle disease virus.

As a prelude to infection of cell cultures preliminary studies on the morphology and ultrastructure of C. sputorum ss mucosalis and C. coli were undertaken. Negatively-stained preparations of serologically representative strains of mucosalis showed the presence of comma, S-shaped and long filamentous forms. Organisms were observed with three different surface coats: i) rough and scaly types with deep transverse clefts, ii) smooth types with longitudinal ridges and iii) smooth types without clefts or ridges. The production of these cell surface structures appeared to depend on the age of the culture and the type of growth medium used. Mucosalis organisms invariably possessed a single polar flagellum and the flagellar appendage showed the presence of a 'collar-like' structure on either side of the filament at the site of attachment to the basal granule.

In thin-sections, the presence of a double layered cell wall and cytoplasmic membrane were clearly visible, the cytoplasmic substance was granular and contained numerous polyphosphate crystals. By comparison, a strain of C. coli showed coccoid or comma-shaped organisms with leathery cell surface with or without transverse clefts, however on section this organism closely resembled C. sputorum ss mucosalis except for the presence of a large cytoplasmic vacuole.

C. sputorum ss mucosalis requires a hydrogen microaerophilic atmosphere for growth and maintenance, without which it becomes non-viable after 10-12 hr. Certain types of cell cultures can support 'parasitic growth' of this organism in the absence of such a hydrogen microaerophilic atmosphere. The evidence suggests that this 'cell dependant' growth is due to intracellular multiplication and release of bacteria from the infected cell cultures. In infected BK cells 'parasitic growth' of mucosalis can persist for up to 7 weeks.

Investigations on the adhesive properties of mucosalis have shown that bacterial attachment is specific and is 'transitory', lasting upto 10-12 hr post-inoculation. There appears to be a direct relationship between the ability of cell cultures to support 'parasitic growth' and to attach mucosalis organisms. The mechanisms involved in the adhesive process are complex although bacterial motility seems to enhance the attachment of mucosalis organisms. Bacterial cell surface

structures, but not the flagella, are involved in the adhesive process to host-cell surface receptors.

Infection of suspensions of trypsinized cells or preformed monolayers with C. sputorum ss mucosalis did not appear to interfere with the initial attachment and/or growth of infected cells, although characteristic cytopathic changes are found during later stages of infection. Electron microscopic studies show that following attachment to cell surfaces bacteria are engulfed by the infected cells and are later found in the phagosomes or free in the cytoplasmic substance. The intracellular fate of mucosalis organisms in the infected cell cultures depends on the type of cell infected. Pig kidney cells appear to destroy mucosalis organisms rapidly and characteristic bacteria disappear from the cytoplasm to be replaced by accumulations of granular material. In contrast, bovine kidney cells infected with mucosalis show, in addition to degenerate forms (ghost cells), morphologically normal bacteria. Comprehensive evidence has been obtained, by viable counts of bacteria, light, immunofluorescent and electron microscopy, which strongly suggests that mucosalis is capable of intracellular multiplication, at least in the BK cell line.

The response of different types of cell cultures to infection with mucosalis differs and can be broadly grouped as following:

i. cell cultures that show 'parasitic growth' of mucosalis and rapid CPE with cell fusion and destruction of the infected monolayers. These include PPK, PK, and PK_{pi} cells.

ii. cell cultures that are less readily destroyed by mucosalis infection and give rise to the production of markedly enlarged 'altered cells', and limited cell fusion. Such cultures include BK, BK_{pi}, OK_{pi}, BHK and HeLa cells.

iii. cell cultures including Vero and $LLCMK_2$ lines, that are 'initially' refractory to 'parasitic growth' and the production of CPE but which, on re-infection, behave like those in group (ii).

iv. cell cultures that normally support 'parasitic growth' but fail to show any CPE, of which DK cells is an example.

v. cell cultures that fail completely to support 'parasitic growth' and which do not show a CPE, even after super-infection e.g. primary cultures of CEF.

The experimental evidence indicates that the cellular abnormalities produced are associated with either the intracellular growth, or the presence of intracellular killed bacteria or bacterial products.

The significance and future application of the findings of this work in relation to the pathogenesis of porcine intestinal adenomatosis, the disease associated with the presence of intracellular C. sputorum ss mucosalis, are discussed.

CHAPTER I

CHAPTER I

REVIEW OF LITERATURE.

INTRODUCTION.

Infectious enteric diseases of swine are important to the veterinary clinician and have been recognised by them for many years. They have an almost world wide distribution and are regarded as a major cause of economic loss to the pig industry. The range of microorganisms that are associated with these intestinal infections in pigs is wide and the agents most frequently incriminated are Escherichia coli (neonatal enteritis), Treponema hyodysenteriae (swine dysentery), Clostridium perfringens type C (necrotic enteritis), various salmonellae (enteritis) and some viruses including the coronavirus of transmissible gastroenteritis.

However, despite extensive investigations and numerous successful experiments in which the diseases have been reproduced with individual organisms, in many instances the disease process remains obscure and the aetiology is complex. The association between specific microorganisms and other enteric diseases is even less clear. This is particularly true of those conditions presenting as a thickened ileum or large intestine in which the primary changes in the mucosa are proliferative (porcine intestinal adenomatosis - PIA), complicated by extensive haemorrhage (proliferative haemorrhagic enteropathy - PHE), or by necrosis (necrotic enteritis - NE) or loss of the mucosa with muscular hypertrophy (regional ileitis - RI).

I.A. HISTORY OF PORCINE INTESTINAL ADENOMATOSIS

Attempts by earlier workers to obtain an understanding of PIA, based on pathological descriptions of the lesions, have contributed little towards its aetiology. Biester and Schwarte (1931) were perhaps the first to draw attention to the adenomatous lesion in the intestines of pigs and again, in 1939, Biester et al., described a rapidly developing intestinal adenoma in a pig. Similar lesions to those described by Biester and his colleagues were observed by Moynihan and Gwatkin (1941) in a six-month-old pig affected with chronic diarrhoea and emaciation.

In Denmark, Emsbo (1951) carried out a detailed study of porcine adenomas and stressed the close similarity between PIA and that of Crohn's disease in man, a view shared by Crohn and Turner (1952). In recent years, PIA has been reported from many parts of the world: in the United States (Biester and Schwarte, 1931), Canada (Moynihan and Gwatkin, 1941), the United Kingdom (Field et al., 1953; Nielson, 1955; Rowland and Rowntree, 1972), Hungary (Korpassy and Tiboldi, 1957), Australia (Pullar, 1958; Kelly and Cameron, 1976), Belgium (Hoorens, 1962), Holland (Westendorp, 1965), Mexico (Necoechea et al., 1969), New Zealand (Cordes and Dewes, 1971), Finland (Rahko and Saloneimi, 1972a, b, c, d; Saloneimi et al., 1972), Sweden (Martinsson et al., 1974)and India (Rajan et al., 1975).

These investigations have resulted in a fairly clear appreciation of the pathology of the condition but, unfortunately, have contributed very little to our under-

standing of the aetiology of the condition. On several occasions transmission experiments have been attempted by workers in different countries. As early as 1929-32, Adersen endeavoured to transmit the disease by feeding minced infected organs or bacterial cultures obtained from the intestine of typical cases, and through contact between healthy and infected pigs. In a similar experiment conducted by Biester and Schwarte (1931), feeding of intestinal contents and scrapings from the large intestine of affected pigs resulted in acute dysentery, and histological examination showed proliferative changes in the epithelium. However, it is difficult to evaluate the significance of these findings since, at that time, workers were largely unaware of the specific clinical disease or aetiology of swine dysentery. Other unsuccessful attempts to transmit PIA with materials from affected animals have been reported by a number of investigators including Biester et al., (1939); Emsbo (1951); Korpassy and Tiboldi (1957) and Rowland and Rowntree (1972).

Although Hoorens (1962) failed to reproduce PIA, by feeding talc and silica gel, and by such surgical procedures as blockage of lymphatics, partial occlusion of the gut lumen and injection of sclerosing agents into the gut wall and lymphatics, he concluded that the obstruction of the lymphatics of the gut wall and mesentery could have an important bearing on the pathogenesis of the condition. This possibility has also been stressed by Kalima (1971) in Finland.

Sclerosing agents have also been used by other workers including Rahko et al., (1973) and Kalima et al., (1976) who considered that granulomatous inflammation with foreign body giant cells in the absence of proliferative changes, was also a sequel of experimental lymphatic obstruction.

The nature of the lesion in PIA and, in particular, the presence of proliferative changes in the epithelial cells resembling an adenoma, tempted many workers to consider the possibility of a virus aetiology (Biester et al., 1939; Korpassy and Tiboldi, 1957). Unfortunately, however, their transmission experiments proved unsuccessful and no evidence of viral infection was obtained.

More recently, a number of viruses including transmissible gastroenteritis virus, vomiting and wasting disease virus and rotaviruses have been shown to be associated with enteric disorders of swine (Rodger et al., 1975; Lecee et al., 1976; Woode et al., 1976), and viruses resembling reoviruses have been isolated from cases of proliferative ileitis and haemorrhagic bowel syndrome, corresponding to PIA and PHE respectively (Bergeland et al., 1975). However, the significance of this last report is not clear since "reoviruses" could not be demonstrated in all cases of PIA and PHE, and were frequently present in a wide variety of other enteric disorders. Similarly, various aetiological factors have been suggested for PIA, PHE, RI and NE but, so far, experimental reproduction of these conditions has not been achieved. Recent claims by Kalima

et al., (1976) and Roberts et al., (1977b) for having reproduced PIA and NE await confirmation.

Perhaps the most significant observation concerning the aetiology of PIA was the demonstration of intracytoplasmic bacteria in the immature proliferative epithelial cells of affected mucosa (Rowland et al., 1973). These organisms were morphologically indistinguishable from vibrios and lay freely within the apical cytoplasm of the epithelial cells. Their identity was confirmed by immunofluorescence staining, electron microscopy and further bacteriological studies (Rowland and Lawson, 1974; Lawson and Rowland, 1974; Lawson et al., 1975b).

This bacterium differs from other campylobacters (vibrios) isolated from pigs and has been designated Campylobacter sputorum ss mucosalis (Lawson et al., 1975b). Although the aetiological significance of C. sputorum ss mucosalis in PIA has not been determined, it is interesting to note that an organism closely resembling mucosalis has subsequently been reported in cases of PIA or PHE in Sweden (Martinsson and Ekman, 1974; Gunnarson et al., 1976) and Australia (Kelly and Cameron, 1976; Love et al., 1977; Love and Love,1977).

B. CLINICAL MANIFESTATIONS IN PIA, NE, RI OR PHE.

Clinical symptoms of PIA and RI are most frequently seen in post-weaned pigs of six - fourteen weeks of age, but cases have also been recorded in older pigs (Emsbo, 1951; Hoorens, 1962; Westendorp, 1965; Rahko and Saloniemi, 1972a). In the post-weaned pig the condition may be suspected by the

presence of anorexia and wasting (Nielsen, 1971; Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Martinsson et al., 1974; Jonsson and Martinsson, 1976), although the course of the disease may be more acute when associated with intestinal perforation and subsequent peritonitis (Blood and Henderson, 1974; Field et al., 1953).

Scouring is not a prominent feature in PIA or RI but when the condition is associated with necrosis of the mucosa resulting in NE, diarrhoea is a likely symptom. The diarrhoeic faeces often contain flecks of necrotic mucous membrane and pigs affected with NE may become gradually more depressed, ending in death.

Biochemical changes reported in PIA include decreased serum albumin (Nielsen, 1967; Martinsson et al., 1974), decreased zinc concentration (Martinsson and Ekman, 1974) and decreased total protein, transferrin and alkaline phosphatase in the serum (Martinsson et al.,1976). Significant increases in the number of white cells and in the serum cortisol and α-antipyrin levels have also been reported (Martinsson et al., 1976).

In contrast to PIA or RI, PHE is more commonly encountered in adult animals although cases can occur in pigs of all ages over three weeks (Rowland and Rowntree, 1972). The onset of the condition is rapid and pigs pass large quantities of black, foul-smelling, altered blood in the faeces. Death is often sudden and in some instances affected animals may be found dead without having shown any clinical signs. A gradual recovery over several days

in some affected pigs has been recorded by Pill (1971) and Rowland and Rowntree (1972).

C. HISTOPATHOLOGY OF PIA COMPLEX.

Hyperplasia and immaturity of the intestinal epithelium have been described as the most important tissue changes in PIA and RI (Biester and Schwarte, 1931; Biester et al., 1939; Emsbo, 1951; Dodd, 1968; Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Jonsson and Martinsson, 1976).

In uncomplicated cases of adenomatosis the thickening of the intestinal mucosa is due to proliferation of the epithelial cells. In addition, the villous pattern is lost and the glands are hyperplastic, elongate and show branching. The epithelial cells are immature with frequent mitotic changes and goblet cells are few in number or absent. There is a sharp demarcation between the affected and surrounding normal tissue. In the large intestine the mucosal thickening once again is due to epithelial proliferation, elongation and increased branching of the glands. As in the small intestine, there is a marked absence of goblet cells in the affected areas. In histological sections, the not uncommon polypoid lesions arising from the mucosa appear to be wider at the surface than at the base and, as in the small intestine, there is clear demarcation between normal and affected areas.

Regional ileitis (RI) is associated with granulation tissue proliferation in the lamina propria and submucosa. The mucosa is irregularly present and the muscle coats are substantially hypertrophic. Scattered islands of remaining

mucosa are composed of immature hyperplastic epithelium. As in PIA, this epithelium contains a large number of bacterial bodies and cultural examination has recovered C. sputorum ss mucosalis (Rowland and Lawson, 1975b; Rowland et al., 1976) from these lesions.

In necrotic enteritis (NE) histological examination of the underlying surviving mucosa of the affected small or large intestine shows hyperplasia with marked immaturity of the cells. Goblet cells are usually absent. Recently, attention has been drawn to the similarities between PIA and NE (Rowland and Lawson, 1975b; Rowland et al., 1976). The intracellular vibrio (C. sputorum ss mucosalis) which is associated with PIA can also be demonstrated in these immature proliferative cells and can readily be isolated from the lesions of NE.

Proliferative haemorrhagic enteritis (PHE) is characterised by thickening of a varying length of terminal ileum and oedema of the associated mesentery. There is marked epithelial hyperplasia in which immaturity of the cells is a feature and only a few goblet cells are present. Bacteria, which morphologically resemble those seen in PIA, have been observed lying freely within the apical cytoplasm of epithelial cells in the affected mucosa (Rowland and Lawson, 1975a, b; Rowland et al., 1976). In addition, positive immunofluorescence using hyperimmune serum prepared against bacteria isolated from cases of PIA, has been reported in PHE lesions (Rowland and Lawson, 1975a). The presence of an intracellular vibrio in PHE has been

described in Australia (Kelly and Cameron, 1976; Love et al., 1977), and it is of interest that Love et al., (1977) have isolated a bacterium from a number of cases of PHE which is morphologically and biochemically indistinguishable from C. sputorum ss mucosalis of Lawson et al.,(1975b).

In this thesis, the term PIA will be used to include those conditions in pigs in which there is thickening of the ileum or large intestine due, primarily, to epithelial hyperplasia and where the lesions are regularly associated with the presence of an intracellular vibrio, namely C. sputorum ss mucosalis.

D. ULTRASTRUCTURE OF PIA.

Extensive studies on the ultrastructure of the affected alimentary tract have been carried out by Rowland et al., (1973); Rowland and Lawson (1974); Martinsson et al., (1974) and Jonsson and Martinsson (1976).

Ultrastructurally, the adenomatous epithelial cells resemble undifferentiated crypt cells. At the surface, the microvilli are poorly developed. Within the cytoplasm the terminal web, the membrane and the endoplasmic reticulum are less elaborate than in the mature normal absorptive cells of the villus. Ribosomes are numerous and variable in size, and impart a granular appearance to the cytoplasm. Electron dense secretory granules are prominent towards the free border. The nucleus and mitochondria present no unusual features.

Affected epithelial cells, in addition to the normal cellular organelles, show curved bacterial bodies lying free

within the apical cytoplasm which are not apparently bound by membranes. However, a halo or zone of clearing may be seen in some preparations but this is generally considered to be an artifact. These bacterial bodies are clearly distinguishable from other organelles in the cells and are occasionally seen undergoing division. They have a vibrio-like morphology and the presence of a distinct cell wall. As mentioned previously the bacterium seen in lesions of PIA was first isolated in culture by Lawson et al., (1975b), who consider it to be different from other known Campylobacter species and named it C. sputorum ss mucosalis.

E. CAMPYLOBACTER (VIBRIO) INFECTION IN THE PIG.

Doyle (1944) first isolated a vibrio from pigs affected with swine dysentery, to which he later gave the name Vibrio coli, (Doyle, 1948). Isolations of similar bacteria from the alimentary canal and faeces of pigs have been reported by a number of investigators including Roberts (1956); Deas (1960); Lussier (1962). Furthermore Doyle (1948) and Roberts (1956) considered this organism to be the causal organism of swine dysentery and claimed to have been able to reproduce the disease in experimental pigs, but several other workers were unable to confirm these findings using pure cultures of V. coli (Warner, 1965; Harris and Kinyon, 1975). At present, there is considerable doubt as to the role of this organism as a cause of swine dysentery (Taylor and Alexander 1971; Harris et al., 1972).

The identification and taxonomic position of this organism has become confused but it seems likely that a

number of catalase positive Vibrio species may on occasion be recovered from pigs some of which are similar to Campylobacter fetus ss fetus (Campylobacter fetus ss intestinalis)* whilst others have the properties of C. coli (Campylobacter fetus ss jejuni)* (Veron and Chatelain, 1973).

More recently, an organism with a vibrio-like morphology was demonstrated in the cytoplasm of immature proliferative epithelial cells in PIA (Rowland et al., 1973) and its identity was confirmed as C. sputorum ss mucosalis (Lawson et al., 1975b). Such organisms have been recovered from the oral cavity of a number of clinically healthy pigs, as well as from the enteropathies RI, PHE and NE, by Lawson et al., (1975a) and Rowland and Lawson (1975b). These observations have subsequently been confirmed by workers in Sweden (Martinsson et al., 1974; Gunnarsson et al., 1976) and Australia (Kelly and Cameron 1976; Love and Love,1977; Love et al., 1977).

The biochemical characteristics of the members of the genus Campylobacter and their taxonomic implications have been discussed by Veron and Chatelain (1973) and Smibert (1978). The features of C. sputorum ss mucosalis indicating that it should be included in the genus Campylobacter have been presented by Lawson et al., (1975b) and Roberts (1978). This organism differs from the majority of other campylobacter isolated from pigs by being catalase-negative. The majority of clinical isolates from PIA belong to a single serotype, A, all of which are agglutinated by antiserum prepared against the type strain 253/72 (NCTC 11000). Another serotype, B, has been associated with adenomatosis and

*Names in parenthesis refer to the current nomenclature as described in Bergy's manual of determinative Bacteriology, 8th Edition (1974).

these two serotypes have distinct surface antigens. Other biochemically or serologically distinct catalase negative campylobacters can be isolated from the alimentary tract of pigs on occasion but in many instances the presence of these organisms is not associated with disease.

II. IN VITRO STUDIES OF PATHOGENIC BACTERIA IN CELL CULTURE SYSTEMS.

A. USE OF CELL CULTURES IN STUDIES OF HOST-PARASITE RELATIONSHIPS.

In recent years cell culture techniques have advanced enormously and have been successfully applied in many different fields of biology. There seems to be no more suitable, realistic, versatile or practical means of studying in vitro the complex natural relationships and mechanisms of intracellular parasitism. Indeed, an in vitro cell system, be it of primary, secondary, established cells or organ cultures, provides a convenient and, sometimes, the only alternative method for investigating the mechanisms of host-parasite relationships of micro-organisms which multiply intracellularly. This is particularly true where infection of the host animal is unsatisfactory for a variety of reasons, or in those disease conditions where suitable experimental animals are not available. Although the use of cell culture systems in the investigation of intracellular parasitism, host-parasite relationships and disease processes has been under severe and constant criticism largely because cultured cells do not truly represent the tissues of origin, the method provides an inexpensive, uncomplicated and

flexible biological testing ground in which the advantages clearly out-weigh the disadvantages.

B. BACTERIAL ATTACHMENT AND GROWTH CHARACTERISTICS IN CELL CULTURES.

As early as 1916, Smyth recognised that infection of relevant host animals did not always allow pathogenic micro-organisms to multiply sufficiently to permit examination of the disease process and that part of this problem might be circumvented by cultivating the organisms in artificial media. Following an investigation of the growth of avian tubercle bacilli in chick embryo explants, Smyth also suggested the possibility of employing tissue explants for differentiating pathogenic from non-pathogenic organisms. The advantages of tissue explants in studies of host-parasite relationships at the cellular level are limited by a variety of factors including unstable cell populations and uncontrolled metabolic activity. Moreover, the response of epithelial cells grown *in vitro* tends to differ from that of cells at the time when the tissue is removed from its animal source.

Interest in experimental infection of cell cultures with bacterial species was renewed when Dulbecco and Vogt (1954) introduced improved techniques for the propagation of viruses in tissue culture monolayers of individual cell types. In recent years the use of tissue culture systems has advanced considerably due, mainly, to the wide range of cell lines that are now available and the ease with which most cell culture systems can be manipulated.

At present, tissue cultures provide one of the most convenient and reliable means of studying the behaviour of microbial agents, particularly with regard to intracellular parasitism and the pathogenic mechanisms involved at the cellular level.

Intracellular parasitism is a biological phenomenon whose fundamental nature is still not clearly understood. In intracellular parasitism associated with viruses and rickettsiae the genome of the parasite is unable to replicate without assistance from the host cell because of restrictive metabolic limitations imparted by inherently deficient structures or membranes. Thus, the complexity of the relationship and the dependance on the host cell vary greatly with the basic nature of the infective agent. Accordingly, intracellular parasites are broadly considered under two groups: the obligate organisms (e.g. viruses, chlamydias and most rickettsias) and the non-obligate (e.g. bacteria).

In order to gain some understanding of the increasingly complex biological relationships associated with intracellular parasitism, most in vitro studies have been carried out in cell or organ cultures as a means of providing near to normal conditions for both the parasite and its host cell. The intracellular behaviour of obligate parasites such as viruses has been well documented but, surprisingly, very little work has been done with other microorganisms, and for this reason the nature of the host cell interactions in bacterial infection is still not fully understood. In

recent years, however, a number of studies of host-parasite relationships of pathogenic bacteria in cell culture systems have been attempted and there has been significant progress in our knowledge of the mechanisms of bacterial attachment to the surfaces of animal cells.

It has been known for some time that bacteria possess adhesive properties but it is only within the last few years that this phenomenon has become widely recognised and accepted as one of the important factors in the initiation of bacterial infections of man and animals. Several pathogenic bacteria display a sharply restricted range of the hosts they infect under natural conditions. This narrow host specificity has hindered studies of the natural ecology of many pathogens, particularly those associated with human infection, because they generally fail to colonize the mucosal surfaces of common experimental animals when inoculated by the oral or respiratory routes. To a great extent, this situation has been circumvented by conducting bacterial adhesion studies in cell culture systems derived from the natural hosts.

The following brief review summarises the investigations that have carried out on various aspects of bacteria-cell culture interactions.

1. MYCOBACTERIA.

In a series of experiments, Shepard (1955; 1957a, b; 1958) considered the growth characteristics of virulent and avirulent mycobacteria in HeLa cells and compared the results with similarly infected monkey kidney and human

amnion cells. Unlike avirulent strains, pathogenic mycobacteria grew much faster and their rates of multiplication in HeLa cells compared well with their pathogenicity for mice and guinea pigs. The bacteria behaved similarly in all the three cell lines, although a distinctly slower growth was observed in the human amnion cells.

Multiplication of Mycobacterium lepraemurium in primary spleen explants (Rees and Wong, 1958) and in spleen cells (Wallace et al., 1958) obtained from animals previously infected with this organism, has been reported. In some respects, the tissues were found to be unsuitable because the cells could not be readily established, maintained or subcultured, and did not provide adequate growth conditions for this slowly multiplying organism. Garbutt et al., (1958) and Wallace et al., (1958) succeeded in growing the rat leprosy bacillus in established cell lines of rat (strain 14pf) and mouse fibroblasts (strain L) respectively. Limited multiplication of the organism was observed in mouse cells, but only with the addition of hydrocortisone at a concentration toxic to the cells. Further, it was noted that some of the media which supported the growth of cell cultures were unsuitable for bacterial multiplication, and this observation was subsequently confirmed by Garbutt (1965). Rees and Garbutt (1962), in a detailed study on the growth characteristics of M. lepraemurium in rat fibroblasts, obtained a continuous growth of the organism for periods of upto 500 days despite regular subculturing of the monolayers. They also observed a gradual increase

in the number of cell-associated bacteria after a few initial subcultures, and more than 80% of the cells were infected with at least 10 or more bacteria after 300 days. It was also of interest that the "cell adapted" bacteria (derived from 154-328 days culture) quickly degenerated when cultured in cell-free media and failed to grow on conventional bacteriological media used for the routine growth of this organism. However, bacteria recovered from a 200-day-old infected cell culture showed growth, although irregularly, when inoculated into fresh fibroblast monolayers. More recently, Brown and Draper (1970) carried out an electron microscopic study of rat fibroblasts experimentally infected with M. lepraemurium but failed to find any structural abnormalities.

2. BRUCELLAE.

Growth in chicken fibroblasts of several strains of Brucella abortus, Brucella melitensis, Brucella suis and their variants, as well as other pathogenic bacteria, has been described by Holland and Pickett (1956). In these experiments, two-day-old monolayers on Formvar coated coverslips were infected with various brucellae (5 hr), group A streptococci, enterobacteria (2 hr) including E. coli, Salmonella typhosa (typhi) and Alcaligenes faecalis. At the end of this period 10 µg/ml of streptomycin was added to the medium to prevent extracellular bacterial growth of Gram-negative bacteria and 10 IU/ml of penicillin was incorporated in media used to support monolayers infected with streptococci.

The results obtained indicated that only brucellae and antibiotic-resistant mutants of S. typhi multiplied in chicken fibroblasts despite the presence of bactericidal concentrations of streptomycin. The authors suggested that the streptomycin sensitive Brucella strains were capable of growing in the fibroblasts whereas the sensitive S. typhi strains were not. Smooth strains of brucellae were ingested and multiplied in fibroblasts while variants, including strain 19, failed to grow unless a massive inoculum was used. B. abortus and B. melitensis were least cytopathogenic while B. suis strains produced cellular granularity, heavy vacuolation and eventual destruction of the fibroblasts. A mixed inoculum of smooth and non-smooth (intermediate and rough) strains of Brucella indicated that the smooth strains were taken up more rapidly by chicken fibroblasts and survived longer than the others. Braun et al., (1951) obtained similar results, but Freeman et al., (1961) found no significant difference between various strains of brucellae. It is difficult to compare these findings since the respective authors used different techniques and strains.

Richardson (1959) reported on the ability of B. abortus to invade and multiply rapidly within primary bovine cell cultures of foetal skin and kidney, as well as in cell cultures from adult uterine mucosa, testes, spleen, bone marrow and lung. Using streptomycin (2 µg/ml) to limit extracellular growth, Richardson observed that the number

of intracellular brucellae depended, within limits, both on the number of organisms present in the inoculum and on the duration of infection of the cell cultures, although this was not necessarily so during the first 72 hours of intracellular location.

Later, employing similar B. abortus - cell culture systems, Richardson and Holt (1962) studied the synergistic action of antibiotics on the intracellular multiplication of the organism. Extracellular concentrations of penicillin at 5-10 µg/ml and tetracycline at 0.5-1 µg/ml were found to be bacteriostatic for intracellular bacteria while streptomycin at 5-50 µg/ml failed to prevent intracellular multiplication. However, 50 µg/ml streptomycin and 50 µg/ml penicillin or 10 µg/ml streptomycin and 0.5 to 1 µg/ml of tetracycline were markedly bactericidal for intracellular brucellae. Further to this study, Richardson and Holt (1964) described the multiplication of B. abortus in primary bovine spleen and lung cell cultures. Viable counts carried out by lysing infected cell cultures indicated intracellular multiplication after an initial drop and immunofluorescence tests on similarly infected cells detected bacterial antigens although it was difficult to assess the extent of intracellular multiplication. Destruction of brucellae was evident in spleen cells and it was assumed that this was brought about by the host cell enzymes.

In a series of experiments involving infection of baby hamster kidney cells (BHK) with strains of B. abortus, Hatten and Sulkin (1966a, b) described the intracellular

morphology of the organism and the production and colony characteristics of brucella L-forms. Coverslip preparations stained immediately after absorption showed that most bacteria adhered around the periphery of the cell until, after 24 hr, the organisms had migrated to a more central location within the cytoplasm and were located singly or in clumps. After this period, pleomorphic forms appeared which, on further incubation, developed into small or large granular bodies which reacted specifically with brucella antiserum in fluorescent-antibody tests.

Brucella L-forms were recovered from the inoculated cell cultures irrespective of the presence of antibiotics in the medium. Of the antibiotics tested, penicillin or streptomycin in concentrations of 5-40 μg/ml did not greatly affect intracellular survival of altered brucellae but a combination of these two antibiotics (2.5 and 2.0 μg/ml respectively) effectively reduced the number of cultures yielding L-forms. By contrast, tetracycline (20 μg/ml) was found to be just as effective as the combination of the higher concentrations of penicillin and streptomycin and various concentrations of this antibiotic (0.1 to 2.0 μg/ml) in combination with 5 μg/ml of penicillin or streptomycin significantly reduced recovery of L-forms. However, in all these situations L-forms were recovered for several days even after the elimination of parent bacteria.

The colonial morphology of L-forms on different types of bacteriological media and their ability to revert to bacterial types appeared to be dependant upon the duration of intracellular survival of the organism. The L-forms

recovered during early periods of cell infection promptly reverted to bacterial types while those obtained after 7 days of intracellular growth virtually failed to do so, suggesting that they had become stable.

Cytological changes in monkey kidney cells (LLCMK2) due to Brucella canis and its L-phase variant have been described by Egwu and Eveland (1979). Preformed monolayers were separately infected with parent, L-phase variant, revertant and a mixture of these strains. Antibiotics were used to limit extracellular growth and to prevent possible reversion of L-phase variants in the cell cultures. Intracellular bacteria were recognised by light microscopy of stained coverslips, immunofluorescence and electron microscopy. The results also showed gross damage to the monolayers (in about 85% of parent; 80% revertant; 55.5% L-phase variant and 70.5% mixture of strains), and cytopathic changes included pyknosis, nuclear disorganisation, cytoplasmic vacuolation, degeneration and destruction.

3. ENTEROBACTERIA.

Shepard (1959) examined the ability of HeLa cells to phagocytose S. typhi and a number of other organisms including Pasteurella (Francisella) tularensis, B. abortus, B. suis, B. melitensis, Mycobacterium tuberculosis, Staphylococcus aureus, group A and group D streptococci, Histoplasma capsulatum and Candida albicans. Coverslips cultures were grown in Eagle's medium containing 20% of either chicken, guinea-pig, rabbit, monkey, dog, sheep, horse or human serum. The inoculated monolayers were incubated at 37°C

for 24 hr, stained with Giemsa and Ziehl-Neelsen and examined. The uptake of the various microorganisms was found to be selectively promoted by the serum from different species of animals. Dog serum enhanced the phagocytosis of S. typhi and F. tularensis; guinea pig serum that of group D streptococci and H. capsulatum; chicken and guinea pig serum that of B. abortus, B. melitensis and B. suis; and guinea pig and sheep serum, S. aureus.

In order to examine the intracellular growth of these organisms, coverslip preparations of HeLa cells were exposed to an "infection medium" containing the appropriate organism and a suitable serum that supported maximum phagocytosis (vide supra). The period of exposure was varied (from 2 hr to 3 days) depending upon the growth characteristics of the organism. Thereafter, the monolayers were refed with fresh medium containing human serum and appropriate antibiotics to limit extracellular growth. Intracellular growth was determined by viable counts and the results showed that the "natural" pathogens multiplied in the cells at rates related to their opti mal growth on bacteriological media, whereas strains of lower pathogenicity grew more slowly.

Showacre et al., (1961) obtained a similar pattern of intra- and extracellular growth of S. typhi in infected L929 mouse fibroblasts. However, unlike Shepard (1959), they observed that the addition of penicillin, synnermatin, streptomycin or chloramphenicol to the medium promptly inhibited both intra- and extracellular bacterial multiplication.

Giannella et al., (1973) compared the invasiveness of several strains of Salmonella typhimurium in HeLa cells and rabbit ileal mucosa with their virulence in mice. The cell culture procedures included inoculation of preformed monolayers of HeLa cells with each of 12 strains of the organism (3×10^7 bacteria/ml) for a period of 5 hr. The coverslip preparations were then rinsed in three changes of balanced salt solution (BSS) to limit extracellular bacterial multiplication and returned to the incubator after refeeding with antibiotic free medium. Monolayers were removed at 0, 3, 7 and 24 hr, rinsed three times in BSS and stained with Giemsa.

Invasion of HeLa cells occurred with eight of the strains examined, as shown by the presence of bacteria in 20-50% of the cells at 7 hr and in 70-90% of cells at 24 hr. This infection resulted in complete destruction of the monolayers. Further, the same "pathogenic" strains also invaded rabbit ileal mucosa whilst the remainder were non-invasive. There was no correlation between virulence in mice and the capacity to invade either HeLa cells or rabbit ileal mucosa. By comparison, a strain of Shigella flexneri known to be invasive in experimental animals, was taken up only in very small numbers by HeLa cells when examined under similar conditions, whereas a non-invasive strain of E. coli had difficulty in multiplying extracellularly.

Kihlstrom and Edebo (1976) described the association of viable and inactivated S. typhimurium (395 MS), and its rough mutant (MR 10), with HeLa cells. They noted that the

rough mutant showed a greater tendency for cell attachment than did its parent strain. Heat and UV-inactivated mutants attached poorly compared with viable bacteria, whereas similar exposures did not alter the behaviour of the smooth parent strain.

Later, Kihlstrom (1977) using the same bacteria-cell culture system, attempted to distinguish quantitatively between attached and engulfed bacteria by indirect immunofluorescence and gentamycin treatment of infected monolayers. Acetone fixation of infected cells allowed the passage of antibodies through the cell membranes and thus permitted recognition of intracellular bacteria by fluorescent antibody staining. Gentamycin poorly penetrated the infected cell, thereby allowing intracellular growth but, at the same time, inhibiting multiplication of extracellular bacteria. Both techniques showed that smooth, as well as rough variants, gained intracellular access, with the latter in greater numbers. Unlike other workers involved in the study of *in vitro* bacteria-cell culture interactions, Kihlstrom recognised that repeated washing of infected monolayers did not eliminate extracellular and cell membrane-attached bacteria. In this context it is worth noting that Gerber and Watkins (1961) and Giannella *et al.*, (1973) used only repeated washing of infected monolayers to eliminate extracellular bacterial growth and that antibiotics were not incorporated in the medium.

Gerber and Watkins (1961) grew strains of *S. flexneri* and *Shigella sonni* in the Henle 407 intestinal epithelial

cell line. Extracellular bacterial growth was controlled by repeatedly washing the infected monolayers and replacing the medium every 2 hr during the course of incubation. All of the strains examined were found to be capable of intracellular multiplication, but the individual growth rates varied. Cytopathogenicity was not evident in cells infected with "low yielding" strains, although cytoplasmic disintegration, pyknosis and hyperchromatosis of the nucleus were features of "high-yielding" shigellae.

Employing similar cell infection techniques, Labrec *et al.*, (1964) studied the growth characteristics of virulent *S. flexneri* 2a and its mutant strain in HeLa cells, as part of a series of *in vivo* experiments involving guinea-pigs and monkeys. The parent strain of the organism was found to be cytopathogenic and produced degeneration of the infected HeLa cells, whereas the avirulent mutant failed to induce cellular abnormalities. Invasion of intestinal epithelial cells of experimental animals was obtained only with the virulent strain.

Apart from the above studies involving the growth characteristics of several species of enterobacteria in various cell culture systems, there are a number of reports describing the adhesive properties of these organisms in isolated epithelial cells derived from their natural hosts. These studies are of particular interest in understanding bacterial pathogenicity since they more closely represent the *in vivo* situation than heterologous cell lines.

Wilson and Hohmann (1974) studied several strains of *E. coli* for their ability to adhere to isolated intestinal

epithelial cells of pigs and concluded that K88 and K88ab antigens were associated with bacterial adhesion which could be neutralized by specific antiserum. In contrast, enteropathogenic strains obtained from cattle or man failed to adhere to pig cells. E. coli strains isolated from urinary tract infections in man adhered to urinary tract epithelial cells, suggesting the importance of bacterial adhesion in pathogenicity (Svanborg-Eden et al., 1977; Svanborg-Eden and Hansson, 1978). Recently, Varian and Cooke (1980) examined in vitro adhesive properties of strains of E. coli isolated from upper and lower urinary-tract infections and from faeces of man, to isolated urinary and buccal epithelial cells and HeLa cells. The results of these studies indicated that pathogenic strains adhered more readily than faecal strains and were also capable of invading HeLa cells. Adherence was most pronounced on urinary epithelial cells, followed by buccal and HeLa cells. Poor adhesion of E. coli and Enterobacter aerogenes to human vaginal epithelial cells in vitro has been reported by Mardh and Westrom (1976). Pilus mediated adhesion of Klebsiella pneumoniae, recovered from human urinary tract infections, has also been demonstrated in vitro using rat bladder epithelial cells, (Fader et al., 1979).

4. FRANCISELLAE -YERSINIAE.

Shepard (1959) reported the phagocytosis and intracellular growth of F. tularensis in HeLa cells and showed that dog serum was particularly useful in the uptake and intracellular growth of this organism.

In a detailed study of F. tularensis infection in

mouse fibroblasts (L-cells), bovine kidney and human amnion cells, Merriot et al., (1961) observed bacterial invasion and growth with virulent strains, whereas less virulent strains did not multiply intracellularly and required a larger inoculum to produce penetration. The presence of intracellular bacteria was compared by viable counts of infected monolayers treated with streptomycin and by immunofluorescence. Cytopathic changes in the infected cells were not observed and bacterial growth was largely influenced by the type and amount of serum used, as well as by other nutrients in the medium.

Intracellular multiplication of Pasteurella (Yersinia) pseudotuberculosis in primary cultures of rabbit spleen and kidney cells has been reported by Richardson and Harkness (1970). A wild-type virulent and an avirulent mutant of this organism showed similar growth rates although their intracellular growths were influenced by the atmosphere, constituents of the medium and amount of bacterial inoculum.

Ingestion, followed by limited survival of Y. pseudotuberculosis in HeLa cells was demonstrated by Bovallius and Nilsson (1975). In these experiments the presence of intracellular bacteria was detected by three different techniques; viable counts on antibiotic treated infected monolayers, indirect immunofluorescence and electron microscopy. Large numbers of organisms were present in cytoplasmic vacuoles and remained viable for at least three days and there appeared to be a direct correlation between these cell-associated bacteria and the size of the inoculum.

The survival of Y. pseudotuberculosis in preformed monolayers of HeLa cells has been investigated by Brunius, (1980). Monolayers were exposed to bacteria for 1 hr., then antibiotics added (100 IU/ml penicillin and 100 µg/ml streptomycin) for a similar period in order to kill the non-phagocytosed organisms. The antibiotic treatment was repeated daily for 1 hr, the cultures rinsed in PBS and then reincubated in antibiotic-free medium. At appropriate intervals infected cells were lysed with sodium deoxycholate and viable counts were made to assess intracellular bacterial growth.

The results of this investigation showed an initial decline in the numbers of intracellular bacteria, followed by a steady increase during the 2nd and 3rd day, suggesting intracellular multiplication. Infected cells showed degenerative changes on the 4th day post-inoculation. Although the author used immunofluorescence staining of unfixed infected cells to demonstrate the absence of attached and extracellular bacteria, it must be questioned if discontinuous antibiotic treatment for 1 hr each day, would have been sufficient to eliminate all extracellular bacteria.

5. GONOCOCCI.

In recent years, gonococcus-cell culture interactions have been the subject of extensive investigations and have contributed significantly to our knowledge of bacterial adhesion to cell surfaces.

A sustained growth (upto 88 days) of Neisseria gonorrhoeae in cultured KB cells (human mouth epidermoid

carcinoma) has been reported by Gavrilescu et al., (1966) and reversion of avirulent gonococcal Kellogg types 3 and 4 to virulent types 1 and 2 occurred after growth for 48 hr in monkey kidney cells (Kenny and Aris, 1969). Although Waitkins and Flynn (1973) were unable to confirm reversion with Kellogg types 3 and 4 in infected 3T3 mouse fibroblasts, Vero or LLCMK2 monkey kidney cells, they observed a progressive increase in the rate of bacterial growth during the first 24 hr of cell infection together with engulfment and the intracellular location of gonococci in ultrathin sections of these cell cultures. In an earlier study on experimental infection in human volunteers, Kellogg et al., (1963), found that types 1 and 2 were pathogenic, while types 3 and 4 were avirulent.

Selective adhesion of N. gonorrhoeae types 1 and 2 to human epithelial cells (Punsalang and Sawyer, 1973), cell cultures (Swanson, 1973; Brodeur et al., 1977) and human sperm (James-Holmquest et al., 1974) has been reported. More recently, Gubish et al., (1979) employing double radio labelling, thin sectioning and scanning electron microscopy of HeLa cells infected with N. gonorrhoeae confirmed the earlier finding that virulent bacteria from colony types 1 and 2 attached more readily than avirulent organisms from colony type 4. In addition they showed that trypsin treatment of HeLa cells reduced the degree of bacterial attachment although the situation was restored 24 hr later. Unlike other workers, they only described the association of bacteria with cell surfaces

and did not observe ingestion of the organism.

Human fallopian tube organ culture proved to be particularly useful as a laboratory experimental model in gaining an understanding of the pathogenic mechanisms of N. gonorrhoeae (Carney and Taylor-Robinson, 1973; Ward et al., 1974; Johnson et al., 1977). The method is ideally suited to the study of gonococcal interactions with mucosal epithelial cells since the fallopian tube is naturally susceptible to in vivo infection (salpingitis) and there is no differentiation of the mucosal cells during the first 1-2 weeks in organ culture. Also the histological relationships between the component tissues forming the fallopian tube remain undisturbed in vitro and, finally, it is possible to monitor the ciliated epithelial cells without disturbing the culture.

It is also of interest that fallopian tube organ cultures seem to reflect, to some extent, gonococcal infections in vivo since there is evidence that commensal strains of Neisseria fail to damage tissue cultures in contrast to those strains of N. gonorrhoeae which destroy mucosal epithelium. The ability of virulent colony type I organisms to produce more rapid destruction of cell cultures than other colony types compares well with the findings reported by Kellogg et al., (1963) with human volunteers.

It has also been shown that greater numbers of N. gonorrhoeae adhere to isolated human vaginal epithelial cells than do Lactobacillus acidophilus, group B streptococci and Corynebacterium vaginale (Mardh and Westrom, 1976),

also that freshly isolated gonococci adhere in greater numbers than laboratory strains, and that adherence increases as the acidity of the culture medium increases.

6. STREPTOCOCCI.

Interest in the microbial flora of the human oral cavity has increased in recent years because of growing evidence that several species of bacteria are the aetiological agents of dental caries and periodontal diseases (Socransky, 1970; Scherp, 1971; Gibbons and van Houte, 1975). Quantitative studies involving different parts of the mouth have led to the recognition that particular sites specifically harbour distinct microbial populations.

Bacterial colonization of various buccal surfaces requires first, that the organisms become firmly attached and second, that they are able to proliferate at the site of attachment. More than any other indigenous bacteria, the study of oral streptococci has provided convincing information on the ecological importance of bacterial adhesion on selective colonization.

A long and detailed series of investigations by Gibbons and his colleagures has resulted in the recognition of four distinct species of oral streptococci (Streptococcus salivarius, Streptococcus sanguis, Streptococcus mitis and Streptococcus mutans) which colonize particular surfaces in the buccal cavity. S. salivarius preferentially colonizes the dorsum of the tongue, S. sanguis and S. mutans predominate on the surface of the teeth and S. mitis inhabits the non-keratinized oral mucosa (Liljemark and

Gibbons, 1972; Gibbons and van Houte, 1975). Their recovery from particular oral surfaces has been clearly demonstrated to be associated with their adhesion to these surfaces (Hillman *et al.*, 1970; Gibbons and van Houte, 1971; Ørstavik *et al.*, 1974). The different species and perhaps strains of oral streptococci, possess different adhesive properties which dictate the habitat of the organisms. The specific mechanisms involved are discussed later.

Unlike gonococci, pathogenic streptococci and staphylococci have not been studied to any great extent in cell cultures although their adhesive properties are well documented. Frost (1975) examined the attachment of *Streptococcus agalactiae*, *Streptococcus faecalis*, *S. aureus*, *E. coli* and *Corynebacterium bovis* to the ductular epithelial cells of the bovine udder. *S. agalactiae* and *S. aureus* were able to adhere in large numbers whereas the other bacteria adhered poorly, if at all, thus emphasising the importance of initial adhesion in the pathogenesis of mastitis due to these organisms. Adhesion of *S. faecalis* to the surface of rat tongue (Gibbons *et al.*, 1976) and that of group A streptococci (isolated from human pharynx and skin) to human oral and skin cells has also been reported (Alkan *et al.*, 1977). Recently, Raza *et al.*, (1980) have shown the ability of *S. aureus* to attach to nasal epithelial cells.

7. VIBRIOS

In recent years a convincing amount of experimental evidence has been put forward to show that the adhesion of vibrios to mucosal surfaces influences colonization and thereby contributes to the virulence of the organisms. For some time it has been recognized that Vibrio cholerae attaches intimately to the intestinal mucosa (Freter, 1969) and that failure to do so results in a reduction of virulence (Fubara and Freter, 1973; Guentzel and Berry, 1975; Schrank and Verwey, 1976). Fluorescent antibody (Labrec et al., 1965; Freter, 1969) and electron microscopic studies (Patnaik and Ghosh, 1966; Nelson et al., 1976) have clearly demonstrated the adhesion of cholera organisms to the epithelial cells of the intestines. Nelson et al.,(1976) have shown that in attaching to rabbit intestine,cholera vibrios become attached at right angles to the surface of cells with their flagellar end free.

Adhesion of V. cholerae to isolated brush borders and to slices of rabbit small intestine has been the subject of investigation by Jones et al., (1976). These studies indicated not only that the organisms readily adhered to brush borders but, also, that the phenomenon was dependant on temperature and required the presence of divalent cations such as calcium. Non-motile vibrio variants lacked the ability to adhere to brush borders but motile revertant vibrios isolated from cultures of such strains were just as adhesive as the original parent strain. It was also found

that vibrios penetrated the intestinal mucous gel and became attached to the microvilli of the epithelial cells, although occasionally they became entrapped in the mucous gel and failed to show attachment.

Adhesion studies on intact slices of rabbit intestine infected with vibrios and Salmonella enteritidis showed a number of important differences. Larger numbers of V. cholerae adhered to mucosal surfaces than to serosal surfaces and further studies suggested that adhesion was related to the presence of at least two types of receptors on the mucosa; and that antibody to the heat-stable antigens of V. cholerae inhibited adhesion. The receptors associated with adhesions of salmonellae appeared to be different from those of V. cholerae, and the adhesiveness of Vibrio parahemolyticus was related to the production of unsheathed lateral flagella (Kaneko and Colwell, 1975).

8. BORDETELLA.

In 1959, Crawford and Fishel cultivated a strain of Bordetella bronchiseptica in KB cells (human mouth epidermoid carcinoma), DMB cells (human non-malignant nasal epithelium), HeLa cells and primary monkey kidney cell cultures. Coverslip preparations of these cells, aged 5-7 days, were infected with the organism for 4 hr and reincubated in media with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) or without antibiotics, in order to evaluate bacterial multiplication.

Although the bacteria failed to multiply in the medium 199 alone or in infected cell cultures incubated up to 12

days in the presence of antibiotics, the monolayers were subsequently overgrown and destroyed by the bacteria 7 to 9 days after removal of the antibiotics. Bacteria grown in cell cultures containing penicillin and streptomycin did not develop resistance to either antibiotics.

Recently, Yokomizo and Shimizu (1979) examined the adherence of B. bronchiseptica, isolated from atrophic rhinitis, to nasal epithelial cells of swine. Organisms in phase I adhered firmly and in large numbers whereas with those in phase III generally showed poor or feeble adherence. Exposure to specific antiserum, heat treatment or pretreatment of bacteria with formalin (1%) significantly reduced adherence, whereas trypsin treatment had little or no effect. Scanning electron microscopy showed adherence of the bacteria to the cilia of nasal epithelial cells.

9. CORYNEBACTERIA.

Attachment of several strains of Corynebacterium renale to the cultured baby hamster kidney cell line (BHK-21) and to primary cultures of dog and rabbit kidney cells was investigated by Honda and Yanagawa (1975). Although bacterial attachment was observed in all of the cell cultures examined, it was found that densely piliated strains attached to a large number of cells (70%) compared with poorly piliated strains which attached to smaller number of cells (30%), and that pretreatment of cell cultures with anti-pilus serum greatly reduced the level of attachment of densely piliated organisms (22%). Electron micrographs of infected BHK-21 cells indicated that the pili were involved in the process of adhesion. Under similar

experimental conditions, a piliated strain of K. pneumoniae showed attachment to all of the cell lines examined, whereas a non-piliated strain of S. aureus failed to do so.

10. LACTOBACILLI.

There appears to be very little information on the growth of lactobacilli in cell cultures, although there are several reports of adhesion to epithelial cell in vivo or in vitro. Attachment of lactobacilli to epithelial cells has been observed in the rat intestine (Brownlee and Moss, 1961), chicken crop (Eyssen et al., 1965; Fuller and Turvey, 1971; Bayer et al., 1975) and pig stomach (Dubos et al., 1965; Tannock and Smith, 1970; Fuller et al., 1978). In poultry, adhesion of lactobacilli appears to be restricted to the crop and has not been observed in the small intestine and caecum (Fuller and Turvey, 1971).

11. MYCOPLASMAS.

Since the first report of mycoplasma contamination of cell cultures by Robinson et al., (1956) it has become apparent that a large proportion of stock cell lines are contaminated with these organisms. The effects of artificial and natural contamination of cell cultures by these agents has been reviewed by Stanbridge (1971). In recent years reports on the association of mycoplasmas with tissue culture cells have been numerous and electron microscopic studies indicate that mycoplasmas reside predominantly in the extracellular environment bound to specific receptor sites on the cell membrane (Edward and Fogh, 1960; Zucker-Franklin et al., 1968). Mycoplasmas have also

been observed in cytoplasmic vacuoles, lying freely within the cytoplasm of necrotic or disintegrating HeLa cells and are sometimes associated with the development of long cytoplasmic processes, particularly in Mycoplasma pneumoniae and Mycoplasma gallisepticum infections (Zucker-Franklin et al., 1966). Marked cytopathic effects including cellular enlargement, the presence of intracytoplasmic inclusions and partial destruction of the monolayers have been reported in primary cultures of calf, pig, and monkey kidney cells infected with Mycoplasma bovigenitalium (Afshar, 1967). Growth studies showed that this organism grew more readily in association with cell cultures than in tissue culture medium alone, and that mycoplasma "toxins" produced similar cytopathic effects to those exerted by living organisms. It is also interesting to note that preinfection of cell cultures with M. bovigenitalium delayed the rate of growth of infectious bovine rhinotracheitis (IBR) virus and the appearance of CPE.

Unlike Mycoplasma hyopneumoniae which failed to produce cytopathic effects in infected primary pig kidney cell cultures, Mycoplasma hyorhinis induced cellular granulation, spindle-cell formation with pyknotic nuclei and the appearance of many coccoid organisms (Pijoan, 1975). Similar cytopathic changes have been described by Potgieter et al., (1972) in synovial fluid cell cultures infected with M. hyorhinis. Recently, Aldridge and Cole (1978) described attachment of Mycoplasma synoviae to chicken embryo fibroblasts and showed by immunofluorescence studies that a

progressive increase in the amount of specific antigen occurred during the first three days of incubation when degenerative changes in the infected cells were imminent. Transmission electron microscopy of infected cells showed close contact between the mycoplasmas and host cell membranes but scanning electron micrographs revealed an uneven distribution of mycoplasmas on the cells of a fibroblast monolayer due, presumably, to differences in the nature and availability of the host cell receptor sites. A similar observation was made by Boatman et al., (1976) in electron microscopic studies on the attachment of bovine mycoplasmas to HeLa cells.

Pathogenic mycoplasmas induced a more rapid loss of ciliary activity in tracheal organ cultures than did attenuated strains (Butler, 1969; Collier et al., 1969; Cherry and Taylor-Robinson, 1970). Recently, Gabridge et al., (1978) used monolayers of hamster respiratory epithelial cells, in place of conventional tracheal organ cultures, to study the effects of M. pneumoniae infection, and found extensive attachment of the organisms resulting in ciliostasis and cytonecrosis.

12. CHLAMYDIAE.

Although chlamydiae have the rare ability to enter and grow within the epithelial and endothelial cells of their hosts, the process of adsorption or attachment of these organisms to tissue culture cells is both inefficient and slow. In order to overcome these difficulties several modifications have been introduced in cell culture

techniques which are particularly useful for primary isolation of chlamydiae from clinical materials. These include centrifuging the inoculum on to the monolayers (Weiss and Dressler, 1960), pretreatment of cell cultures with DEAE-Dextran (Rota and Nichols, 1971) or X-irradiation (Gordon et al., 1972). More recently, however, Johnson and Hobson (1976) have found that normal, non-irradiated McCoy cells are especially suitable for this purpose.

Unlike other pathogenic bacteria, chlamydiae have been extensively investigated in cell cultures systems and a considerable amount of evidence based on the initial interactions of these organisms with tissue cells grown in vitro, suggests that the type of host cell determines not only the number and species of organisms taken up by the cell but also the sequence of events leading to their satisfactory growth and development and, ultimately, the release of infectious progeny particles. Thus, Chlamydia psittaci was found to be capable of entering L-cells by passive phagocytosis without the organisms themselves playing any active role in the process (Friis, 1972). It has also been shown that the uptake of some strains of C. psittaci can be stimulated by DEAE-Dextran treatment (Spears and Storz, 1977) whereas tryptic digestion of L-cells destroys their ability to do so (Byrne, 1976). A similar stimulatory effect on the entry of trachoma strains (Chlamydia trachomatis) into HeLa cells following DEAE-Dextran treatment, compared with the inhibitory action of neuraminidase, has also been reported. However, these

treatments do not appear to affect the growth of lymphogranuloma venereum strains of chlamydia (Kuo and Grayston, 1976).

In contrast, mild heating of the chlamydial inoculum results in inhibition of phagocytosis of C. psittaci in L-cells (Byrne, 1976) and C. trachomatis in HeLa cells, thereby underlining the importance of heat-labile surface components of the parasite as mediators in the phenomenon of phagocytosis. Further evidence in this direction has come from the work of Byrne and Moulder (1978) who used C. psittaci and C. trachomatis to demonstrate "parasite-specified" phagocytosis by HeLa and L-cells. They also suggested that the surface ligands of the parasite, in conjunction with those on the host cell surface, bring about attachment and subsequent phagocytosis. It was also found that the ligands are readily denatured by heat, may be masked by specific antibody and are resistant to proteases and detergents; whilst the host cell surface components are reversibly removed by proteases, and treatment with cycloheximide inhibits chlamydial phagocytosis. Thus, ingestion of Chlamydia by non-professional phagocytic cells such as HeLa and L-cells appears to be mutually dependent on the surface components of the host cell and the parasite.

By employing mouse fibroblasts (L-cells) and repeated exposure to C. psittaci (strain 6BC), Byrne (1978) showed that the initial rates of attachment and ingestion increased in accordance with the multiplicity of infection, but that phagocytosis ceased even though many chlamydial

cells remained in suspension and were available for attachment to the host cell monolayers. For this reason, he suggested that attachment and ingestion of large numbers of chlamydiae might cause simultaneous injury both to the plasma membrane itself and to the metabolism of the host cell. Support for this view was provided by scanning electron micrographs of monolayers of L-cells inoculated with high multiplicities of Chlamydia which showed direct damage to the host cells with rounding up and detachment from the glass (Moulder et al., 1976; Byrne, 1978).

13. RICKETTSIAE.

From the very early days of tissue culture, several investigators have grown rickettsias in tissue explants and, with the rapid advances made in modern cell culture methods, quantitative assays of the rickettsial infection cycle have been made possible (Bozeman et al., 1956; Schaechter et al., 1957).

In recent years a number of detailed studies on various aspects of rickettsia-host cell interactions have been reported. Burgdorfer et al., (1975) described the early destruction of host cells and the rapid spread of Rickettsia rickettsii in tissue culture monolayers, and similar observations were made by Wisseman et al., (1976) on chicken embryo fibroblast and L-929 cell cultures. In addition to the rapid spread of infection, Wisseman and colleagues found that a substantial number of organisms accumulated extracellularly rather than in the cytoplasm,

while the presence of compact intranuclear masses in some of the cells suggested that R. rickettsii was capable of penetrating the nucleus from the cytoplasm.

Although the intracellular growth cycle of several species of rickettsiae has been extensively investigated, very little information is available concerning the changes that occur in the fine structure of the infected tissue culture cells. Anderson et al., (1965) reported the growth of several rickettsiae in the cytoplasm and, in some cases, in the nucleus of BS-C-1 monkey kidney cells but were unable to find a correlation between the cell changes and infection. On the other hand, Silverman and Wisseman (1979) described rapid changes in the morphology of chicken embryo fibroblasts infected with R. rickettsii. In electron micrographs of infected cells several progressive host cell lesions were seen. These included widespread dilation of the rough endoplasmic reticulum and outer nuclear envelope, associated with accumulation of electron dense material within the cisternae of the intracellular membranes. The rickettsiae were found free in the host cell cytoplasm, or within the invaginations of the nuclear envelope, but not within the cisternae formed by the swollen endoplasmic reticulum. As a result of intracisternal swelling and fusion of intracellular membranes during the later stages of the infection cycle, the majority of rickettsiae were surrounded by host cytoplasm, bound by host-derived internal membranes, and appeared to persist in this state until cell lysis occurred.

By contrast, the effect of prolonged intracellular growth of Rickettsia prowazekii in chicken embryo fibroblasts appeared to be less dramatic (Silverman et al., 1980). Cytoplasmic changes were not apparent until late in the intranuclear growth cycle when cells, heavily laden with large number of rickettsial bodies, began to rupture. The only recognisable changes in heavily infected cells, before lysis occurred, was condensation of the interstitial matrix of some mitochondria and the apparent dissociation of ribosomes from the rough-surfaced endoplasmic reticulum. Morphological changes in the infecting rickettsiae were apparent by about 40 hr post-infection and consisted primarily of increased electron density, slight reduction in size and vacuolation of the cytoplasm.

C. MECHANISMS OF BACTERIAL ADHESION TO HOST CELLS.

a. Introduction.

Although some bacteria are introduced mechanically into the host by means of vector bite or trauma, most infections begin on the mucous membranes of the alimentary, respiratory or urogenital tracts. These surfaces are regularly protected in a non-specific manner by movements of the luminal contents, surface mucus and commensal organisms (Smith and Pearce, 1972; Smith, 1976). The initial interactions between bacterial cells and mucous surfaces are generally considered to be one of three types: i) attachment and multiplication without significant penetration, as seen in cholera and whooping cough; ii) attachment, penetration and multiplication in the mucosal cells without further

systemic invasion, as seen in bacillary dysentery and iii) attachment and penetration systemically through or between the mucosal cells, as seen in salmonellosis and streptococcal infections (Smith and Pearce, 1972; Schlesinger, 1975; Arbuthnott and Smyth, 1979).

Despite the widespread occurrence of bacterial attachment to tissue surfaces in many natural environments, surprisingly little is known about the mechanisms responsible for these initial interactions. However, in recent years, numerous attempts have been made to elucidate the nature and distribution of the surface components of parasites and host cells that are associated with adhesion.

The surface components of microorganisms can contribute to infection of mucosal surfaces by i) promoting adherence to the host cell surface, thus resisting the mechanical flushing action of the moving luminal contents, ii) overcoming competition from the normal and commensal flora for sites on the mucosa, and possibly by affording protection from the effects of antimicrobial substances produced by them (e.g. fatty acids,), iii) protection of the organisms from immunoglobulins and phagocytic cells derived from mucous membranes, and by promoting either the penetration of epithelial cells or multiplication *in situ* thereby facilitating spread to other tissues (Smith and Pearce, 1972; Schlesinger, 1975).

Pathogens and potentially pathogenic commensals attach to mucous surfaces with considerable selectivity. For example, enteropathogenic strains of *E. coli* adhere to the

ileal rather than to the duodenum of pigs and calves, and to the epithelial cells near the villous tips rather than the base (Arbuckle, 1970; Smith and Pearce, 1972). In the human oral cavity, S. mutans and S. sanguis adhere to the teeth and S. mitis to the buccal mucosa (Gibbons and van Houte, 1975). This selective adherence of bacteria to host cells involves specific interactions with the cell surface components about which little is known. However, there are a number of examples where some knowledge has been obtained of the factors responsible for bacterial adhesion to host cells and these are reviewed in the following section.

1. ENTEROBACTERIA.

The properties of Enterobacteriaceae that enable them to adhere to host cells in vivo or in vitro have been discussed in an earlier section of this chapter and it is emphasised that the factors responsible differ in individual species of bacteria.

Fimbriae or pili occurring on the surface of several species of enterobacteria are the structures primarily associated with bacterial adhesion. These short, straight and hair-like bacterial appendages were named fimbriae (Latin, thread) by Duguid et al., (1955) or pili (Latin, hair) by Brinton (1965) but other names have also been proposed. Ottow (1975) recommended that the term fimbriae be reserved for structures, other than flagellae, and sexpili. In this study, the terms "fimbriae" or "pili" are considered to be synonyms but preference is given in the text

for the name used by the individual worker.

Duguid et al.,(1966) recognised seven types of common fimbriae in enterobacteriaceae. The commonest, type 1, which is present in most species, e.g. E. coli, enables the bacterium to adhere firmly to different types of animal cells, including red blood cells, as well as epithelial cells of the alimentary, respiratory and urinary tracts (Duguid, 1968). Serologically, type 1 fimbriae from different strains of E. coli are closely related, if not identical (Gillies and Duguid, 1958; Nowotarska and Mulczyk, 1977).

The precise function of type 1 fimbriae is difficult to assess since they can be produced by saprophytes such as Klebsiella aerogenes, commensals including E. coli and pathogens such as salmonellae. Whether or not their function is the same in each of these ecologicallly different groups remains to be established. Fimbriae of types 2 and 3 are devoid of adhesive properties for many types of cells although they are able to agglutinate several species of animal red blood cells, whereas type 4 fimbriae are believed to have adhesive properties for some bacteria e.g. Proteus mirabilis to renal cells (Silverblatt, 1974).

Specific binding of purified type 1 pili of E. coli and agglutination of guinea-pig erythrocytes has been reported by Salit and Gotschlich (1977) and Brinton (1965). That this effect is inhibited by D-mannose suggests that the surface sugars on host cells act as determinants of adhesion. (River and Darekar, 1975; Ofek et al., 1977; Salit and Gotschlich, 1977).

In addition to classical "Duguid-type" fimbriae, E. coli are known to produce a number of different fimbrial antigens that mediate bacterial adhesion and colonization of intestinal epithelial cells. Many strains of E. coli that are enteropathogenic for young ruminants produce a plasmid-determined proteinaceous fimbrial antigen designated as K99 (Ørskov et al., 1975) which is responsible for colonization of the small intestine (Sojka, 1971; Smith and Linggood, 1972; Ørskov et al., 1975; Meyers and Guinee, 1976). In addition the K99 antigen is present in some enteropathogenic E. coli strains that cause diarrhoea in neonatal pigs (Moon et al., 1977). Protection of lambs (Sojka et al., 1978); piglets (Morgan et al., 1978) and calves (Nagy, 1980) against colibacillosis has been achieved by inoculating pregnant dams or young animals with purified cell-free K99 pilus antigen and specific antibodies to this antigen have been demonstrated in colostrum and serum.

Yet another fimbrial antigen designated 987P has been demonstrated in some enterotoxigenic piglet strains of E. coli not possessing either K88 or K99 pili, and bacterial adhesion to the small intestine is thought to be mediated through this antigen (Nagy et al., 1976, 1977; Issacson et al., 1977). Unlike K88 and K99 fimbrial antigens, the 987P antigen has not been shown to be plasmid controlled (Walker and Nagy, 1980) and is not associated with haemagglutination.

The fimbrial antigens responsible for colonization of the human intestine have been designated CFA/I and CFA/II

and are produced by specific serotypes of enterotoxigenic strains of E. coli. These antigens are plasmid-mediated surface-associated fimbriae or pilus-like structures which enable CFA-positive E. coli to adhere to the epithelial cell surface of the small intestine as a prerequisite to establishment and multiplication. (Evans et al., 1975, 1978; Evans and Evans, 1978). The CFA/I and CFA/II are mannose-resistant haemagglutinins which are serologically distinct from each other and from common fimbriae (mannose-sensitive haemagglutinins) possessed by many strains of E. coli (Evans et al., 1977, 1979). The presence of a plasmid-determined adherence factor in enteropathogenic E. coli has also been reported by McNeish et al., (1975) and Williams et al., (1977).

The production of non-fimbrial adhesions, also known as "fibrillar adhesins" has been observed in some strains of E. coli and Salmonella. These may also be present in bacteria that produce fimbrial adhesions (Jones and Rutter, 1974), and are responsible for mannose-resistant haemagglutination.

The most thoroughly investigated of these "adhesive antigens" is the proteinaceous, plasmid-controlled K88 antigen produced by a number of enteropathogenic porcine strains of E. coli. This was the first fibrillar substance to be demonstrated and defined in vivo and in vitro. The K88 antigen enables positive strains ($K88^+$) to adhere to the epithelial brush-borders of piglet intestines thereby allowing the bacterium to colonize the mucosal surface,

while negative mutants ($K88^-$) are unable to do so and are non-pathogenic (Smith and Linggood, 1971; Jones and Rutter, 1972). Immunization of piglets with K88 antigen prevents bacterial adhesion and subsequent colonization (Rutter and Jones, 1973), and *in vitro* experiments involving isolated intestinal brush borders indicate interference with or absence of initial attachment (Rutter *et al.*, 1976).

Although the fimbrial and non-fimbrial adhesions of *E. coli* have been well characterized in recent years (Jones, 1977) very little is known about the nature of the receptors on intestinal brush borders Gibbons *et al.*, (1975) have suggested that an oligosaccharide of the host cell glycocalyx consisting partially of a hexosamine residue, might play a significant role as host cell receptors for the K88 antigen. These host cell surface receptors are genetically inherited and certain strains of pigs do not have them (Sellwood *et al.*, 1975). Such animals are resistant to infection as $K88^+$ *E. coli* strains fail to attach to the intestinal epithelial cells.

2. GONOCOCCI.

Electron micrographs of mucosal cells obtained from the urethra of patients with early gonorrhoea show that the gonococci (*N. gonorrhoeae*) were attached and partially embedded in the surface of epithelial and mucus secreting cells (Ovcinnikov and Delektorskij, 1971; Ward and Watt, 1972). Our understanding of the intial interactions between gonococci and host cells has been made possible by the study

of infected human fallopian tube organ cultures (Ward et al., 1974; McGee et al.,1976). In this model, gonococci were found initially to interact with the microvillous projections on the host cell surface which appear to twist toward the invading bacterium and are particularly well developed on the surface nearest the bacteria. Observations also suggest that microvillous formation is stimulated by the gonococci which ultimately become enfolded by the microvillous processes. In due course, resorption of these processes brings the organisms into intimate contact with the host cell surfaces and subsequent phagocytosis.

Gonococci preferentially infect the columnar epithelium of the endocervix and posterior male urethra (Harkness, 1948) which sites are only transiently colonized by neisseriae other than N. gonorrhoeae (Sparkes et al., 1977). Gonococci have a specific affinity for human tissues whilst their reaction with the genital tissues of other species varies. Taylor-Robinson et al., (1974) have shown that piliated and non-piliated gonococci could infect human fallopian tube organ cultures but failed to adhere to the mucosal surface of rabbit oviduct. In contrast, Tebbutt et al., (1976) reported that this was not true for the guinea pig because gonococci attached to the cervix, uterus and male urethra although the animal is resistant to gonococcal infection. However, it seems that different techniques were followed by Tebbutt and his colleagues since the majority of gonococci in their studies were only loosely attached to the guinea pig cells and were

washed off easily, suggesting that high affinity receptors were not involved in this attachment.

The agglutination of human erythrocytes of all blood groups and absence of agglutination of red blood cells of seven other mammalian species by piliated gonococci suggest that there may be some selectivity for human membrane receptors without involvement of the major blood group antigens. Adhesion of piliated gonococci to human spermatozoa and inhibition of attachment by ATP (adenosine triphosphate) and cAMP (Adenosine 3':5' cyclic monophosphate) has been reported by James et al., (1976). They considered that ATP and cAMP altered the polarity of the sperm membrane thus affecting the surface charge. Neuraminidase treatment of cells, as well as binding of plant lectins and antibodies to cell surface determinants, alter cell surface charges and may affect microtubule function, thus interfering with attachment. At present there is no firm evidence to explain the host or organ specificity of attachment sites.

Considerable work has been carried out on the gonococcal surface structures involved in attachment. Primary isolates of gonococci from human patients are invariably piliated although this property is quickly lost in non-selective subculture (Jephcott et al., 1971; Swanson et al., 1971). These primary isolates invariably produce piliated colonies of types 1 and 2. Mutants defective in pilus production are generally of reduced virulence and give rise to colonies of types 3 and 4 (Kellogg et al., 1963). There is now abundant evidence to show that pili facilitate the attachment of gonococci to human cells in tissue culture (Swanson,

1973; Ward and Watt, 1975) and to human spermatozoa (James et al., 1976), while scanning and transmission electron microscopy of human fallopian tubes perfused in vitro with gonococci have shown the organisms adhering to the mucosal surface by bundles of pili (Ward et al., 1974). Although pili are not essential for adherence, they confer up to an eight-fold advantage in attachment to cell membranes depending on the model used (Ward and Watt, 1977). Pearce and Buchanan (1978) found greater attachment of purified ^{125}I labelled pili to buccal epithelial cells at a lower pH (pH 4.5) than pH 7.5. Recent work by Lambden et al., (1980, 1981) suggests the presence of two distinct types of pili, designated α and β. Both types of pili labelled with ^{125}I showed striking differences in their pH dependance for attachment to human buccal cells. The binding or adherence of α-pili was greatest at pH 6.5 (44%) whereas β-pili showed maximum binding at pH 4.5 (13%) and at pH 8.5 (4%). This work clearly raises the the possibility that gonococci are able to produce different pili in vivo according to the physiological conditions present at the mucosal surfaces. Removal of sialic acid residues from buccal cell surfaces by treatment with neuraminidase markedly inhibited the binding of α-pili but had a minimal effect on β-pili. These findings suggest the α and β pili bind specifically to different receptors on buccal cells.

A similar sialic acid - carbohydrate complex on the host cell receptor was suggested by Buchanan et al., (1978),

who also considered that the outer membranes of gonococci play an important part in specific adhesion of non-piliated gonococci to human buccal cells. Modification of the surface membranes of buccal cells by enzyme and chemical treatment greatly reduced the binding of ^{125}I labelled purified pili but did not affect the adhesion of non-piliated gonococci. These findings suggest that specific attachment of non-piliated gonococci is mediated by a surface protein which has been designated protein II (Lambden et al., 1979).

Attachment of gonococci to mucosal cell surfaces appears to be a complex, multifactorial process involving pili, outer membrane adhesions and host cell surface receptors. The mechanisms of gonococcal adhesion, including the role of non-specific factors which influence the process of attachment, are fully discussed by Ward and Watt (1977) and Watt (1980).

3. STREPTOCOCCI.

Streptococci have been extensively studied because of their suspected importance as aetiological agents of human dental caries, and several in vitro and in vivo approaches have been used to investigate their mechanisms of attachment to oral surfaces. The subject has been well reviewed by Gibbons and van Houte (1975) and Gibbons (1980), who emphasise that the mechanisms of streptococcal attachment are not wholly understood and that several factors are probably involved.

S. mutans causes dental plaques and adheres to the

teeth in a two-step process. First, there is a weak reversible association with salivary glycoproteins which form a pellicle on the teeth (Gibbons and van Houte, 1975; Schlesinger, 1975). This is followed by stronger attachment through the production of two sticky glucose polymers (glucans) which are synthesized from dietary sucrose. Mutants lacking the ability to produce glucans are poor producers of dental plaques in pathogen-free and gnotobiotic rats (Smith, 1976). Similarly, enzymes that hydrolyse 1-6 or 1-3 linkages in glucans reduce adherence (Gibbons and van Houte, 1975; Smith, 1976).

The factors responsible for the adherence of S. sanguis to the teeth, and that of S. salivarius and S. mitis to different sites in the buccal cavity, are less well understood, although involvement of a bacterial cell surface fibrillar coat, which is believed to be a lipoprotein, has been suggested (Gibbons et al., 1972; Liljemark and Gibbons, 1972; Lai et al., 1973). This surface fibrillar coat, which has also been found on Streptococcus pyogenes (Swanson et al., 1969), appears to differ from the pili or fimbriae of Gram-negative organisms in that they are shorter, thinner and more widely distributed over the bacterial cell surface. Several studies have shown that these fibrils mediate the attachment to epithelial surfaces of S. salivarius (Gibbons et al., 1972), S. mitis (Liljemark and Gibbons, 1972) and S. pyogenes (Ellen and Gibbons, 1974). In addition, adherence of S. pyogenes to the epithelium of the throat is probably mediated through the bacterial cell wall M-protein

(Ellen and Gibbons, 1972; Schlesinger, 1975) since its removal by means of trypsin or anti-M-protein serum inhibits streptococcal attachment (Ellen and Gibbons, 1972; Smith, 1976).

4. LACTOBACILLI.

Lactobacilli show a high degree of specificity for chicken crop epithelium, and electron microscopy indicates that adhering lactobacilli possess an extra layer on the outer surface of the bacterial cell wall which is not normally seen unless special staining techniques are used. As with other bacterial species, the adhesiveness of lactobacilli is related to the fibrillar nature of this "microcapsule" although it is recognised that adhesion can occur in the absence of fibrils which are not demonstrable in cultures during the stationary phase of growth (Brooker and Fuller, 1975, 1977; Fuller and Brooker, 1980).

The carbohydrate nature of fibrillar adhesion has been identified by chemical and lectin characterisation studies, while the presence of Concanavalin-A (Con-A) receptors on the surface of adhesive, but not on non-adhesive, bacterial cells has been confirmed by selective inhibition of bacterial attachment to epithelial cells previously treated with Con-A, (Fuller, 1975).

In a recent report, Barrow *et al.*, (1980) described the attachment of *Lactobacillus fermentum* strain 14 and *S. salivarius* strain 3/2, to porcine epithelial cells and suggested that the presence of a bacterial microcapsule with fibrils extending to the epithelial cells was characteristic

of the organisms' adhesiveness. They also showed that the extracellular material on the cell walls of these two bacterial species was dissimilar in its chemical composition.

5. VIBRIOS.

V. cholerae colonizes the mucosal surface of the human small intestine and this phenomenon appears to be of importance in the pathogenesis of cholera (Freter, 1969). The mechanisms involved in this process appear to be complex and the organism is believed to produce several adhesins (Jones, 1980).

Studies on the kinetics of colonization of intestinal villi, using scanning and transmission electron microscopy, have revealed that the initial interaction of the vibrio occurred with mucus (Schrank and Verwey, 1976; Jones et al., 1976), followed by adhesion to the brush-borders of the epithelial cells (Guentzel and Berry, 1975; Nelson et al., 1976). The motility of vibrio organisms is a prerequisite for interaction with mucus and this activity may lead to either entrapment within the mucus (Schrank and Verwey, 1976) or to adhesion to the mucus (Freter and Jones 1976). Attachment of vibrios to brush-borders appears to be more complex and flagella are necessary although the polar flagellum does not appear to act as the adhesive appendage (Jones and Freter, 1976; Nelson et al., 1976). In addition, the attachment of vibrios to the brush-borders can be reduced or eliminated in several ways; for example, the absence of bacterial motility at low temperatures, the absence of diavalent cations or the presence of L-fucose.

There appear to be at least two specific mucosal receptors for V. cholerae, one of which is fucose-sensitive on the brush-border epithelium, and another which is fucose-resistant and is located on the intact mucosa at an undetermined site. Bacterial motility is believed to play a role in the transport of vibrios to their fucose-resistant receptors and to facilitate penetration of the intestinal mucus layer (Jones et al., 1976). It is of interest that the flagella of vibrios are sheathed structures (Follet and Gordon, 1963) and in the mouse experimental model, naked (unsheathed) flagella have not proved to be highly protective as a vaccine against oral challenge with virulent vibrios, whereas crude flagella provided complete protection (Eubanks et al., 1977). The latter observation suggests that sheath material may thus act or be a carrier of one of the bacterial adhesins.

However, Freter and Jones, (1976) have shown that the somatic antigen may also be important in adhesion and that the predominant mechanism of association with mucosal surfaces was a chemotactic response of the vibrios to "attractants" produced by the tissue. This process was not, to any noticeable extent, the result of the adhesion of vibrios to the brush-border surfaces. Whether or not adhesion and chemotaxis are components of a continuous interaction remains to be resolved (Freter et al., 1977). Electron microscopic observations which show that the vibrios attach to the epithelium at a pole, with the flagella directed outwards into the mucus and subsequently

by direct contact between the bacterial cell body and the microvilli (Nelson *et al.*, 1976), seem to substantiate the findings of Freter and others (*vide supra*).

6. MYCOPLASMAS.

Pathogenic species of mycoplasma can adhere to leucocytes, tissue culture cells and the surfaces of tracheal organ cultures (Stanbridge, 1971). Some mycoplasmas have distinct organelles at one pole of the bacterial cell (Collier and Clyde, 1971; Wilson and Collier, 1976) and electron microscopy has demonstrated that these organelles are located immediately adjacent to the cell when mycoplasmas attach to cell surfaces and probably act as the adhesins (Collier and Clyde, 1971; Muse *et al.*, 1976). The adhesins of mycoplasmas are not identical and evidence suggests that several other factors may be involved in the adhesive activities of this organism (Sobeslavsky *et al.*, 1968).

7. CHLAMYDIAE.

Chlamydiae are obligate intracellular parasites and attachment to host cell membranes is ,therefore, an integral step in their life cycle. Species of chlamydiae may differ in their adhesive properties to tissue culture monolayers (Kuo and Grayston, 1976; Kuo *et al.*, 1972, 1973) and sialic acid residues on the surface of host cells are believed to act as receptors for *C. trachomatis* organisms, at least under conditions of centrifuge-assisted infection of cell monolayers (Weiss and Dressler, 1960).

Recently, it has been suggested that centrifuge-assisted infection depends on the induction of a host cell-membrane response and that pressure-induced deformation of the cell surface, which is absent on static monolayers, is responsible for the attachment of C. psittaci and, perhaps, for other species also (Allan and Pearce, 1977; Allan et al., 1977).

III. A. CONCLUDING REMARKS.

There can be no doubt that bacterial adhesion plays a vital role in the pathogenesis of many bacterial infections in man and animals. It is also true that much of our understanding of host-parasite relationships stems from the recent advances in cell culture techniques and their application to studies of pathogenic microorganisms. The availability of transmission and scanning electron microscopy has also opened up new dimensions in this important field of study and the interactions taking place between the parasite and its host cell are now better explained than at any previous time.

The use of cell cultures to explore the mechanisms of bacterial pathogenicity has been sadly neglected in the past, but recent progress in this field is beginning to yield valuable information. One of the main disadvantages of cell culture systems in the study of bacteria–host cell interactions is that extracellular bacterial growth tends to persist and is often associated with degenerative changes in the infected cell culture monolayers. In Virology, however, extracellular growth of viable virus particles is not possible and, consequently, considerable

progress has been made in understanding the growth and development of viruses *in vitro* and the mechanisms involved in the pathogenesis of viral infections.

Although the difficulties arising from extracellular bacterial multiplication can often be circumvented by incorporating appropriate antibiotics in the cell culture media, there is a marked difference of opinion about the ability of antibiotics to penetrate host cells and inhibit or destroy intracellular bacteria. The question is a crucial one since the available information strongly suggests that with the possible exception of certain obligate bacteria, sustained intracellular growth in cell cultures is a myth rather than a reality. On the other hand, it is generally agreed that intracellular growth over a limited period is possible with some bacteria at least.

Despite these difficulties, the knowledge gained has led to better understanding of bacterial pathogenic mechanisms, and studies of bacterial attachment have confirmed the importance of initial adhesion in bacterial tropism, colonization and virulence. Identification and characterisation of specific factors involved in bacterial adhesion, such as K88, K99, 987P, CFA/I and CFA/II, is likely to be hard won but the potential rewards in terms of prevention and treatment of disease are clearly considerable. Vaccines that can disrupt bacterial adhesion and colonisation are urgently required and, indeed, there is evidence that use of pilus antigens (K88; K99) as a vaccine is effective.

Apart from these practical considerations, bacteria-host cell interactions have generated a great deal of fundamental interest in the morphology and biological significance of microbial surface components and it is to be hoped that the progress made in this area will encourage similar interest in their counterparts on the host cell.

B. PURPOSE OF THE PRESENT INVESTIGATION.

Ever since the demonstration of intracellular C. sputorum ss mucosalis in adenomatous epithelium (Rowland et al., 1973) considerable doubt has remained about the part played by this organism in the production of these abnormal intestinal cells.

In the past, numerous unsuccessful attempts have been made to reproduce PIA in gnotobiotic, colostrum deprived, conventional neonatal or post-weaned piglets with cultures of mucosalis or diseased tissues (Roberts, 1978; Dr Lawson, personal communication). The results of these experimental procedures have shown that the alimentary tracts of these animals are not generally colonised by mucosalis although the organism may be recovered from this site for a few days after inoculation. Although gnotobiotic animals can become extensively colonised with mucosalis, this does not appear to result in cellular parasitism or morphological changes of the intestinal epithelium.

In view of these observations it was considered necessary to investigate the behaviour of this organism in cell culture systems as an alternative to animal

experimentation, since this *in vitro* approach might throw some light on the mechanisms involved in the pathogenesis of the disease condition. It was also thought that observations on the interactions of *mucosalis* with cultured cells might yield knowledge leading to improvement or modification of the experimental methods in future attempts to transmit the disease in the pig.

In this work emphasis has been placed on obtaining an overall assessment of the interactions of *mucosalis* organisms with cell cultures rather than a detailed investigation of a particular aspect of the relationship between *mucosalis* and the host cell.

CHAPTER II

CHAPTER II

GENERAL MATERIALS AND METHODS.

I. MEDIA AND REAGENTS.

1. Bacteriological media.

The following media were used:

i. Columbia blood agar (CBA) medium was prepared by incorporating 7% defibrinated horse blood into Columbia agar base (Oxoid, CM 331).

ii. Blood agar slopes consisted of 30 ml of CBA in 100 ml medical flats.

iii. Tryptose phosphate broth (TPB) was prepared from tryptose phosphate broth base (Oxoid, CM 283).

iv. Diphasic media consisted of a CBA slope with an overlay of 20 ml of tryptose phosphate broth or a similar quantity of Dulbecco's minimum essential medium (MEM) containing 10% heat inactivated calf serum.

In all cases, Oxoid media were prepared according to the manufacturer's recommendations.

2. Cell culture media.

Dulbecco's modification of Eagle's medium (Dulbecco and Freeman, 1959), Eagle's minimum essential medium (MEM) with Hanks' salts (Eagle 1959) and Medium 199 with Earle's salts (Morgan et al., 1950) were obtained from Gibco-Biocult, Paisley, Scotland, and were supplemented with sodium carbonate (4.4% W/V solution) and L-glutamine (200 mM) at final concentrations of 0.16% and 1.0%

respectively, Also used was Eagle's MEM with Earle's salts modified for suspension cultures (S-MEM), supplemented with L-glutamine and 0.3% methyl cellulose.

Pooled calf or foetal calf serum sterilized by positive filtration through a Millipore membrane filter of 0.22 µm A.P.D. and inactivated at 56°C for 30 min was added to all media at 2.5% for maintenance and 10% for growth, unless stated otherwise.

3. Reagents.

Trypsin.

Initially, 1% trypsin (Difco, Detroit) solution was prepared by dissolving trypsin overnight at 4°C in Dulbecco's PBS and sterilised by filtration through sintered glass. This was stored at -30°C in 200 ml quantities. Trypsin (0.05%) was prepared from the stock solution in PBS and sterilised by sintered glass filtration and stored at 4°C in 200 ml quantities.

Saline trypsin Versene (STV).

This contained 0.01% of 1:250 trypsin (Difco, Detroit, U.S.A.) and 0.01% Versene (Ethylenediamine tetraacetic acid) in PBS (pH 7.2).

Buffer (PBS).

Dulbecco's phosphate buffered saline (pH 7.2) was purchased as ready to use tablets from Oxoid, London.

Erythrocytes.

Fowl red blood cells were obtained by venepuncture

from healthy unvaccinated adult hens and treated with sterile 3.8% sodium citrate to prevent coagulation. Stock suspensions of 1% were prepared in PBS after three washes and stored for 3-4 days at 4°C. Red blood cells collected from guinea pigs, cattle, pigs or horses were similarly processed.

II. BACTERIOLOGICAL TECHNIQUES.

1. Bacterial strains.

In this present work, the investigations of the growth characteristics of C. sputorum ss mucosalis in cell cultures were mostly confined to the proposed type strain 253/72 (NCTC 11,000).

However, in order to obtain a comparative evaluation of the behaviour of this organism in various types of cell culture systems, parallel experiments were generally made with a number of other strains of mucosalis (982/76, 512/77 and 1075/78). In addition selected experiments were carried out using a biochemically and serologically distinct catalase-negative strain of Campylobacter (20/74), C. coli (124/73 A4) and two strains of E. coli of porcine origin.

All of these cultures were obtained from Dr G.H.K. Lawson, Department of Veterinary Pathology, University of Edinburgh, and their particulars are shown in Table 1.

TABLE 1

SPECIES OF BACTERIA EXAMINED IN CELL CULTURES.

Species	Strain	Serotype	Source	Reference
C. sputorum ss mucosalis	253/72*	A	PIA	Lawson and Rowland (1974).
C. sputorum ss mucosalis	982/76	B	PIA	Roberts et al., (1977a).
C. sputorum ss mucosalis	512/77	C	PHE	Lawson et al., (1979).
C. sputorum ss mucosalis	1075/78	A	PIA	Dr Lawson, personal communication.
C. coli	124/73/A4**	-	pig colon	Lawson and Rowland (1974).
Campylobacter sp.	20/74***	-	pig faeces	Lawson et al., (1976).
E. coli	-	0149:K91,K88ac	pig intestine	Dr Lawson, personal communication.
E. coli	-	0149:K85,K88ac	pig intestine	" "

* = Proposed type strain of subspecies mucosalis (NCTC 11,000).

** = A catalase-positive organism with biochemical characteristics intermediate between C. foetus ss intestinalis and jejuni. For convenience this organism will be described as C. coli 124/73 A4 until such time as the taxonomy of this group of bacteria is clarified.

*** = A catalase-negative organism, biochemically and serologically distinct from mucosalis strains.

2. Cultivation of campylobacters.

Columbia horse blood agar plates were inoculated with C. sputorum ss mucosalis, C. coli or Campylobacter strain 20/74 and, after piercing the sides of plastic Petri plates, the cultures were placed in a McIntosh and Fildes' anaerobic jar without a catalyst. The jar was evacuated to a negative pressure of 650 mm of mercury, re-checked after 10 minutes and filled with hydrogen. Ten per cent of this atmosphere was removed and replaced with carbon dioxide prior to incubation at 37°C. Bacterial cultures were maintained in the hydrogen microaerophilic atmosphere and subcultured at weekly intervals.

3. Cultivation of other bacteria.

Columbia horse blood agar plates inoculated with enteropathogenic E. coli were incubated at 37°C and were subcultured at weekly intervals.

4. Source of Campylobacter antiserum.

Hyperimmune rabbit serum prepared against viable cells of C. sputorum ss mucosalis strain 253/72 (Lawson and Rowland, 1974) was obtained from Dr Lawson. This antiserum has an agglutinating titre of approximately $^{1}/_{2560}$ in whole cell agglutination tests with the homologous strain.

III. CELL CULTURE TECHNIQUES.

1. Source of cell cultures.

Ten continuous cell lines, together with primary cultures of pig kidney cells (PPK) and chicken embryo fibroblasts (CEF) were used in this study. The cell lines

included porcine kidney (PK, Stice, 1955), bovine kidney (BK, Madin and Darby, 1958), canine kidney (DK, Madin and Darby, 1958), monkey kidney (Vero and LLCMK2, Hull et al., 1956), baby hamster kidney (BHK-21, McPherson and Stoker, 1962) and human cervical carcinoma (HeLa, Gey et al., 1952) cells. Also studied were lines of porcine (PK_{pi}), bovine (BK_{pi}), and ovine (OK_{pi}) kidney cells persistently infected with Newcastle disease virus (Fraser et al., 1976).

2. Cell culture procedures.

i. Continuous cell lines.

Cells were cultured in 6" x $^{5}/_{8}$" Pyrex test tubes, 4 oz medicinal flats, Brockway "Saniglass" cell culture bottles and one litre Roux flasks. The volume of the medium used in each case was 1, 10, 50 and 100 ml respectively, and the cells were seeded at 10^5 cells per ml unless stated otherwise.

The cells were grown in Dulbecco's minimum essential medium (MEM) with 10% heat inactivated calf serum and, for maintenance, the serum was reduced to 2.5%. Antibiotics were not incorporated in growth or maintenance media.

All cell cultures were reseeded every 7-10 days at which time the cells were detached from the glass with STV solution. The cells were pelleted at 300 x g, resuspended in growth medium and counted in a Neubauer counting chamber before seeding.

The monolayers were incubated for 3-4 days at 37°C and were refed with maintenance medium. Cell lines were

routinely treated once a month with 200 µg/ml of Kanamycin as a precaution against contamination with mycoplasmas.

ii. Primary cell cultures.

Primary pig kidney cell cultures.

Kidneys from a young piglet were collected in MEM. After removing the capsule, the kidneys were rinsed in warm PBS to remove any blood clots and the kidney tissue in the cortical region was chopped up very finely with sterile scissors and transferred to a 200 ml Pyrex bottle containing 150 ml of sterile 0.25% trypsin. The mixture was held at 37°C and stirred by a magnetic stirrer for approximately 2 hr. After 15 min, the supernatant solution was removed, a further 150 ml of trypsin was added and the stirring continued. A second and possibly third discard was made every 15 min until disintegration of the cortical tissue was achieved. The cells were harvested every 30 min, until no kidney fragments remained. On several occasions trypsinization was continued at 4°C overnight.

The suspension of cells was deposited at approximately 500-700 x*g* for 3 to 5 min and the clear supernatant fluid containing the trypsin was discarded. The cells were then suspended at a concentration of 1 ml packed cell volume in 200 ml MEM growth medium. Cells harvested after overnight trypsinization were similarly treated. The suspension of cells was dispensed in Roux flasks and incubated undisturbed for 24-48 hr at 37°C. Monolayers were refed every third day with growth medium and reseeded every 10-12 days after

treatment with STV solution.

Chicken embryo fibroblasts (CEF).

CEF were prepared from one-day-old chicken embryos. The head, limbs and viscera were removed aseptically and discarded. After washing in warm PBS, the tissues were rinsed and subjected to treatment with 0.25% trypsin in PBS at 37°C for 10 minute periods, with intermittent agitation. When the undigested tissues had settled, the supernatant was removed and fresh warm trypsin solution added.

The first two harvests were discarded and fluids from subsequent trypsinizations were stored in cold sterile calf serum at 4°C until the whole series of harvests were collected. The cells were then centrifuged at 300 x g for 10 min and suspended in growth medium containing 10% calf serum. After counting the number of viable cells, the culture vessels were seeded with 5×10^5 cells per ml.

IV. EXAMINATION OF MONOLAYERS.

1. Light microscopy.

Unfixed monolayers were routinely examined by direct microscopy in an Olympus inverted microscope. Also examined were monolayers, fixed in methanol or acetone, and stained by Giemsa's or Giminez's methods.

i. Giemsa staining.

Flying coverslips were fixed in methanol, immersed in a $^1/_5$ solution of Giemsa's stain for 10 min and then differentiated in buffer (pH 6.8) for a similar period. The stained monolayers were then dehydrated through a

series of acetone and acetone-xylol mixtures, cleared and mounted in Depex.

ii. Gimenez's staining (Gimenez, 1961).

Coverslip preparations were fixed in acetone, stained with buffered carbol fuchsin for 3-5 min and washed thoroughly in tap water. The primary stain was decolourized using fast green solution (0.8%) for 10 sec. Dehydration and mounting procedures were similar to those described above.

2. Ultraviolet light microscopy.

Fluorescent antibody staining.

Monolayer cultures on "flying" coverslips were washed twice in PBS, lightly blotted, rinsed and fixed in ice-cold acetone for 10 min and then air-dried at room temperature for 15 min. Dried coverslips were either stained immediately or stored in small tightly stoppered Bijou bottles at -20°C. The staining technique was performed according to the standard indirect method (Nairn, 1976) using an appropriate dilution ($^1/_8$ in 0.01 M PBS; pH 7.2) of rabbit anti-mucosalis serum (strain 253/72, OH-antiserum). The coverslips were immersed in diluted serum and incubated at 37°C for 30 min. The monolayers were then repeatedly washed in PBS by continuous flooding and aspiration for 15 min. After gentle blotting, the coverslips were then stained with an appropriate dilution of fluorescein-labelled anti-rabbit globulin, prepared in sheep, incubated and washed as before. The coverslips were mounted in glycerol-PBS buffer (9:1) and examined in a Zeiss photomicroscope by

ultraviolet illumination.

In some experiments, the coverslip preparations were fixed in 10% neutral formaldehyde for 30 min, air-dried for a similar period and stained as described above.

3. Electron microscopy.

i. Negative-staining.

This procedure was used routinely for staining bacterial cultures grown on Columbia blood agar (CBA), diphasic medium or in cell cultures. In CBA cultures the surface growth was removed at appropriate times with a platinum loop and carefully suspended in a small quantity of prewarmed (37°C) sterile distilled water to give a uniformly thin suspension of organisms. In the case of diphasic cultures, bacterial growth was suitably diluted with warm sterile distilled water prior to examination.

When organisms derived from infected cell cultures were to be stained, appropriate amounts of the supernatant fluid were first centrifuged for 2-3 min at 1500 x g to deposit the cell debris and then recentrifuged for 10 min at 10,000 x g to pellet the bacteria. The supernatant fluid was discarded and the pelleted bacteria were resuspended by gentle agitation in warm sterile distilled water.

The specimen to be stained was placed on a carbon-coated formvar film supported on a 400 mesh coppergrid and, after 30 sec, the excess fluid was removed by careful blotting. The grid was then rinsed in a further drop of distilled water and the excess material removed by gentle blotting. The preparation was then stained for 10-20 sec

with one drop of one of the following: potassium phosphotungstate (2%, pH 6.3); potassium silicone tungstate (2%, pH 7.1); uranylacetate (2%, pH 4.5) or methylamine tungstate (Faberge and Oliver, 1974) (EM scope Ltd., England). Excess stain was removed with good quality absorbent tissue and the preparation was air dried on filter paper or held in an evacuated dessicator over phosphorous pentoxide before being examined at 100 KV in a Philips EM 400 electron microscope.

On occasion, suspensions of selected bacterial strains in warm distilled water were mixed with an equal volume of 1, 5 or 10% neutral formaldehyde for 2 min, prior to negative staining as described above.

ii. Ultrathin section.

Cell cultures.

Following removal of the medium, cell cultures were rinsed in sterile normal saline and fixed in situ with freshly prepared, ice-cold 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for 1 hr at 4°C. The cells were then rinsed in several changes of cacodylate-sucrose buffer* and scraped off the glass with a "rubber policeman" and held in fresh buffer overnight at 4°C. After several washes in PBS, post-fixation was carried out in 1% osmium textroxide in PBS. This was followed by dehydration in a graded series of alcohols and the cells were finally embedded in Araldite. After sectioning the cells on an OM 4 Reichert microtome (Reichert, Austria), they were stained with saturated uranylacetate in 50% ethanol and lead citrate (Reynolds, 1963) and examined in a Philips EM 400 electron microscope at

* Dr Bingham, personal communication.

100 kv.

Bacteria.

One-day-old bacterial growth on Columbia blood agar plates was fixed *in situ* with 2.5% glutaraldehyde cacodylate buffer for 1 hr at 4°C. After several washes in cacodylate-sucrose buffer, the bacterial growth was gently removed by means of a platinum loop and processed for electron microscopy in a similar manner to that described for cell cultures.

CHAPTER III

CHAPTER III

MORPHOLOGY AND ULTRASTRUCTURE OF C. SPUTORUM SS MUCOSALIS AND C. COLI.

GENERAL INTRODUCTION.

The morphology of the bacterial cell has been a fundamental aspect of microbiology ever since Antony van Leeuwenhoek first described his 'animalcules' during the later part of the 17th century and the subject has been extensively investigated by microbiologists using increasingly sophisticated instruments. Knowledge of the morphology and ultrastructure of microorganisms has advanced considerably with the introduction of electron microscopy which has contributed greatly to our understanding of the pathogenic mechanisms of bacteria.

Clearly an appreciation of the ultrastructure of Campylobacter species would yield information of considerable value in achieving the objectives of this work. C. sputorum ss mucosalis has been a recent addition to this genus (Lawson and Rowland, 1974) and, except for its demonstration within the adenomatous intestinal epithelium of the pig (Rowland and Lawson, 1974) and limited cultural studies (Roberts, 1978), the details of the morphology and ultrastructure of this organism have not been fully investigated. However, electron microscopic studies of some other species of the genus Campylobacter, namely Vibrio fetus (Campylobacter fetus ss fetus), Campylobacter fetus ss intestinalis and Campylobacter jejuni (Campylobacter fetus ss jejuni) have been

reported (Rhoades, 1954; Werner et al., 1961; Werner, 1963; Ritchie et al., 1966; McCoy et al., 1975; Pead, 1979).

This study was undertaken so that the author could become familiar with the ultrastructural features of mucosalis grown in culture. This was essential in order to be able to appreciate those features of the organism which might be involved in its attachment to cultured cells, and to recognise abnormalities in structure which might occur as a result of intracellular growth.

b. Design of experiment.

i. Negative-staining of bacteria.

In this experiment, mucosalis strains 253/72, 982/76, 512/77 and 1075/78, as well as C. coli strain 124/73 A4, were obtained either from 1, 2, 4 and 7-day-old CBA cultures or from diphasic growth, and were negatively-stained with different types of heavy metal salts. The surface growth of bacteria was removed by a platinum loop and gently suspended in a small quantity of prewarmed (37°C) sterile distilled water, prior to negative-staining (Chapter II). In addition, a portion of the bacterial suspension prepared from one-day-old cultures was separately mixed with an equal volume of 1, 5 or 10% neutral formaldehyde and held for about 2 min before being negatively-stained.

Whenever diphasic cultures were used the fluid medium was suitably diluted with distilled water prior to negative-staining, but they were not examined after treatment with formaldehyde.

ii. Thin-sectioning of bacteria.

One-day-old surface growths of mucosalis strain 253/72 and C. coli strain 124/73 A4 on CBA plates were fixed in situ with 2.5% glutaraldehyde cacodylate buffer and prepared for thin-sectioning as described in Chapter II.

The negatively-stained and thinly-sectioned specimens of mucosalis and C. coli were examined in a Philips EM 400 electron microscope at 100 KV.

c. Results.

i. Negatively-stained bacteria.

Preliminary examination of C. sputorum ss mucosalis and C. coli organisms stained with different heavy metal salts showed that potassium phosphotungstate, potassium silicone tungstate and uranyl acetate tended to produce a 'positive staining reaction' due, perhaps to penetration of the salt into the bacterial cells. By comparison, methylamine tungstate invariably stained bacterial cells 'negatively' and, for this reason, was used routinely in this study unless stated otherwise. Although certain batches of methylamine tungstate became contaminated with extraneous bacteria after prolonged storage at $4^{o}C$, this problem was overcome by passing the stain through membrane filters of 0.22 μm A.P.D. prior to its use in negative staining techniques.

Morphology of mucosalis organisms.

Mucosalis organisms of all four strains when examined by negative staining showed a very similar morphology and consisted of comma, S-shaped or long filamentous forms of 1 to 5 μm in length and 0.4 to 0.5 μm in width. All three

morphological types occurred in 24 to 48 hr CBA or diphasic cultures but the long filamentous forms usually predominated in old cultures. The S-shaped and filamentous bacteria did not show cellular division and appeared to be composed of undivided or single bacterial cells since they lacked septation on their surfaces (Figs. 1A-B).

Three distinct types of outer cell surfaces or cell coats were observed. The bacterial cells obtained from 24 to 48 hr-old CBA cultures presented a rough scaly cell surface usually with one or more deep transverse clefts, but old CBA, as well as one-day-old diphasic cultures, consisted mostly of organisms with a regular surface with three or more thick longitudinal ridges. Transverse ridges were invariably absent. A third type of organism with a smoother surface, but with neither clefts nor ridges, was observed in 7-day-old cultures. Although most of the organisms in a stained preparation showed one particular type of 'cell coat', small numbers of bacteria of each of the other cell surface types were always present. The periphery of the bacterial cells remained free of any globular or other extensions from the cell wall (Figs. 2A-B-C).

During the first 24-48 hr of incubation the majority of bacteria (over 80%) showed evidence of a single polar flagellum, but variations in the number and distribution of flagella were not encountered. The flagellum measured 5 to 12.5 μm in length and 13 to 18 nm in width and generally appeared to be 1½ to 2-3 times the length of the bacterial cell, but did not show the presence of either a sheath or a clearly defined internal substructure (Fig. 3C).

The flagellum appeared to emerge from a small well defined compact 'button-hole' depression at one pole of the bacterial cell and maintained a uniform diameter along its length except towards the distal extremity which was slightly expanded. Several isolated flagella were observed particularly in diphasic cultures and seemed to be composed of the long filamentous portion with two lateral flap-like structures and a basal granule. The filament appeared to be anchored to the basal granule and, at the point of insertion, a lateral flap-like structure was present on either side giving the appearance of a 'collar'. The basal granule appeared to be more compact and cup-shaped (Figs. 3A-B).

Morphology of C. coli.

Examination of 24 to 48 hr-old CBA cultures of C. coli showed that the organisms were pleomorphic and consisted of comma, S-shaped and coccoid cells (0.7 to 2.5 μm in length and 0.4 to 0.7 μm in width). Long filamentous forms were not observed during this period and the cell surface of most organisms had a 'leathery', wrinkled appearance with prominent deep transverse clefts or fissues (Figs.6A-B). However, older cultures obtained either from the surface growth of diphasic fluids were usually smoother and less 'leathery' and the transverse fissures were poorly defined.

Almost all C. coli organisms possessed single bipolar flagella but occasionally two or three flagella were seen at each pole. The flagella were unsheathed and measured 5 to 15 μm in length and 13 to 18 nm in width but showed no clearly discernible internal substructure. The site of flagellar attachment to the cell wall was surrounded by a

concave depression and contained a basal granule which was often difficult to recognise distinctly in negatively-stained preparations. Isolated flagellar structures were not encountered in cultures of C. coli and could not, therefore, be compared with those of mucosalis organisms (vide supra).

Effect of formaldehyde treatment on the morphological features of mucosalis and C. coli organisms.

Formaldehyde treatment (1%) of mucosalis organisms derived from a 24 hr-old surface growth did not affect the appearance of scaly and rough forms of bacteria. By contrast, higher concentrations (5 or 10%) of formaldehyde detached the outer cell surface structures which were clearly visible as approximately hexagonal structures and are, perhaps, responsible for the characteristic scaly and rough appearance of the cell surfaces. The remainder of the bacterial cell showed that the outer layer of the cell wall had generally 'peeled off' and had separated from the inner layer, to reveal contraction of the cytoplasmic mass (Figs. 4A-B-C).

By comparison, 10% formaldehyde treatment of C. coli produced changes that were restricted to the coat which appeared to be 'fuzzy' and therefore lacked the typical smooth and 'leathery' outer cell surface.

ii. Thinly-sectioned bacteria.

Ultrastructure of mucosalis organisms.

Thin-sections of one-day-old mucosalis strain 253/72 confirmed earlier electron microscopic evidence of the presence of comma, S-shaped and filamentous forms. The

outer bacterial cell wall which was wavy and tightly enclosed around the cytoplasm, appeared to be responsible for the characteristic 'topology' of this organism. The cell wall was composed of two separated electron dense layers and the cytoplasmic membrane also appeared to be double-layered with an intermediate electron-lucent zone (Figs. 5A-B).

The bacterial cytoplasm was uniformly dense and granular but the only intracytoplasmic structures that could be recognised were a large number of electron-dense, darkly-staining spherical particles which appeared to be identical with those described as 'polyphosphate crystals' in C. fetus ss fetus by Rhoades (1954); Werner et al., (1961); Werner (1963) and Ritchie et al., (1966). These structures measured 10 to 60 nm in diameter. Other intracytoplasmic structures were not clearly discernible in the thin-sectioned preparations and, perhaps, this was due to the dense and granular nature of the cytoplasm.

The internal structure of the bacterial flagella could not be seen in these preparations. Most of the organisms showed varying lengths of flagellar stubs attached to their poles, and large numbers of widely scattered pieces of flagellarfilaments lay freely outwith the cells. These preparations confirmed the presence of a single polar flagellum originating in a small cup-shaped depression, but other structures were not clearly visible.

Ultrastructure of C. coli organisms.

Thin-sections of C. coli also showed the comma, S-shaped and coccoid forms described earlier in this chapter. In most cases, the cell wall appeared to envelop the cytoplasmic mass more loosely than was the case with mucosalis thereby producing a clear space between these two structures. The wavy cell wall was double-layered, at least in transverse sections of bacteria, but was less recognisable in longitudinal sections. The cell wall measured 15 to 33 nm in thickness.

The cytoplasmic membrane appeared also as a double-layered structure which tightly invested the cytoplasmic mass and measured 10 to 20 nm across. The bacterial cytoplasm was very densely granular and contained numerous polyphosphate crystals. These electron-dense, darkly staining spherical particles were much larger and more numerous than those present in mucosalis organisms but the nuclear material, or other intracytoplasmic structures could not be identified. Of particular interest in this study was the presence of a large cytoplasmic 'vacuole' lying immediately below the region of flagellar attachment. This structure varied in size and was invariably found at only one end of the bacterial cell (Fig.7A-B).

Large numbers of detached flagellar pieces were seen in thin sections and often it was not possible to demonstrate the bipolar location of flagella on the bacterial cell. Flagella, when present, showed no characteristic internal substructure.

Fig. 1A

One-day-old culture (CBA) of C. sputorum ss mucosalis showing comma, S-shaped and filamentous forms. The majority of the organisms show a rough, scaly outer surface or cell coat, a feature prominently seen in young cultures. Methylamine Tungstate (META) X 15000.

Fig. 1B

A preparation of C. sputorum ss mucosalis obtained from a four-day-old CBA-MEM diphasic culture. The organisms show smooth surfaces with or without characteristic longitudinal ridges. META X 18750.

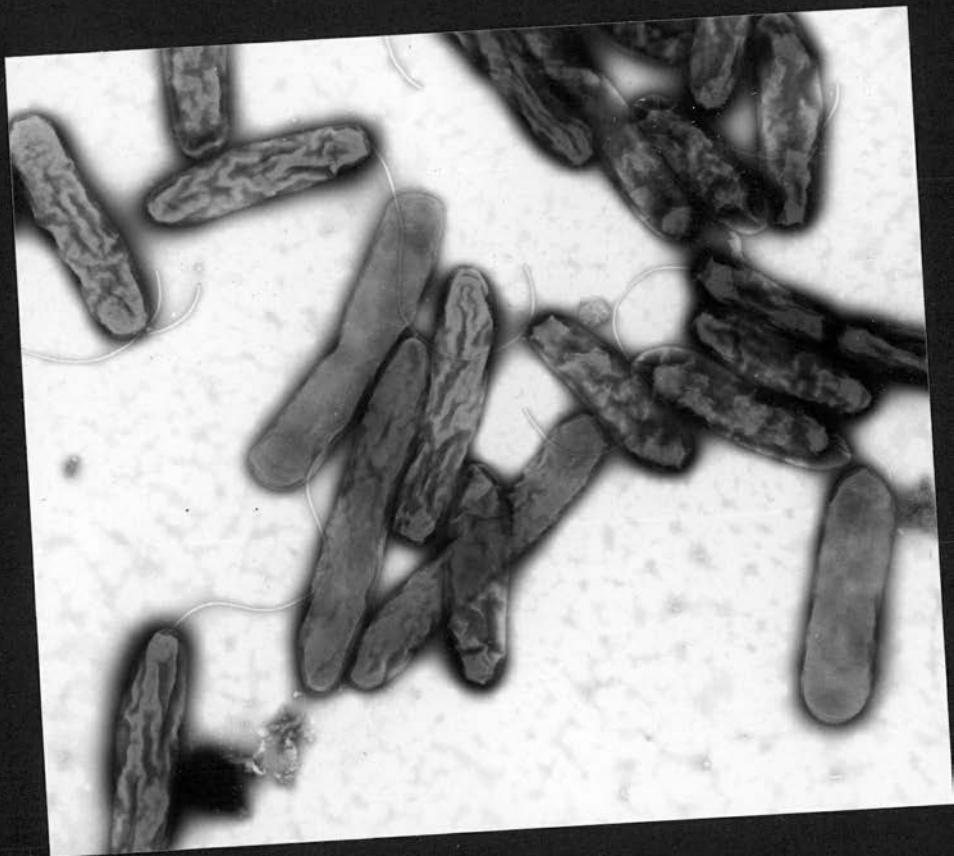


Fig. 2A

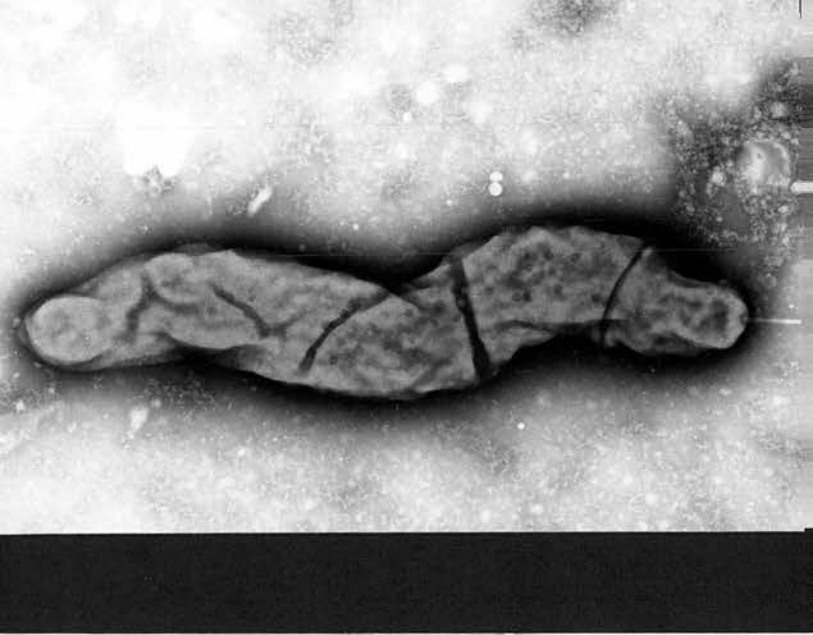
Morphological features of C. sputorum ss mucosalis grown in CBA-MEM diphasic medium (24 hr-old). Note the smooth surface and deep transverse clefts. META X 56250.

Fig. 2B

A similar preparation to that shown in Fig. 2A after 48 hr incubation. The cell surface shows longitudinal ridges but deep transverse clefts are invariably absent. META X 56250.

Fig. 2C

Culture of C. sputorum ss mucosalis. After 7 days' incubation in CBA-MEM diphasic medium the organism has a smoother cell surface, without any scales or ridges, compared with those shown in Fig. 2A or 2B. META X 32250.



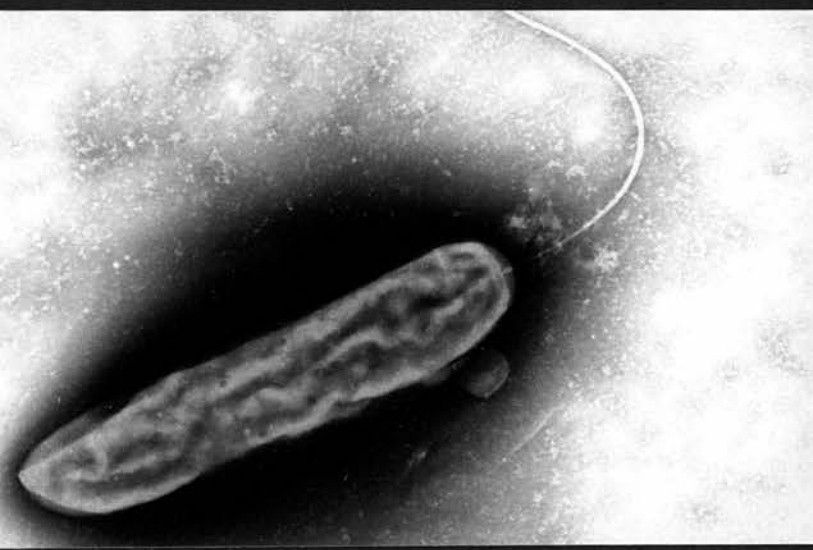


Fig. 3A

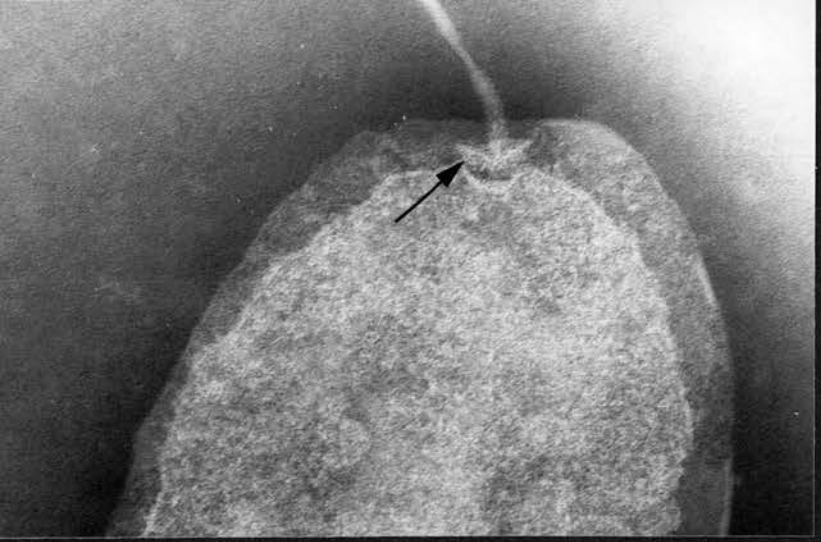
Electron micrograph of a four-day-old culture of C. sputorum ss mucosalis strain 253/72 grown in CBA-MEM diphasic medium. Notice the flagellar appendage and its attachment to the bacterial cell. The lateral 'collar-like' structure (arrow) located just above the basal granule is clearly seen. Potassium phosphotungstate (PTA) X 150000.

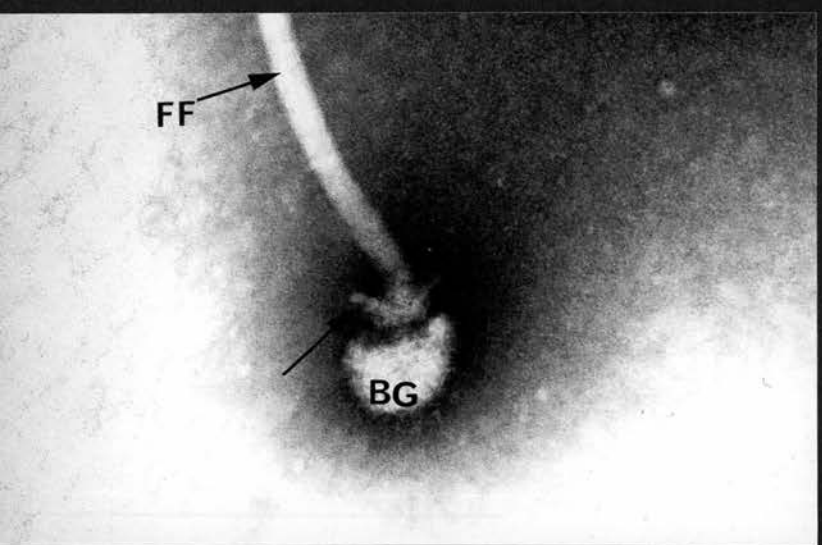
Fig. 3B

An isolated flagellar appendage of C. sputorum ss mucosalis strain 253/72 consisting of a cup-shaped basal granule (BG), lateral collar (arrow) and flagellar filament (FF). PTA X 97500.

Fig. 3C

Flagellar filaments of C. sputorum ss mucosalis. There is no evidence of an internal sub-structure. META X 32250.




FF
BG

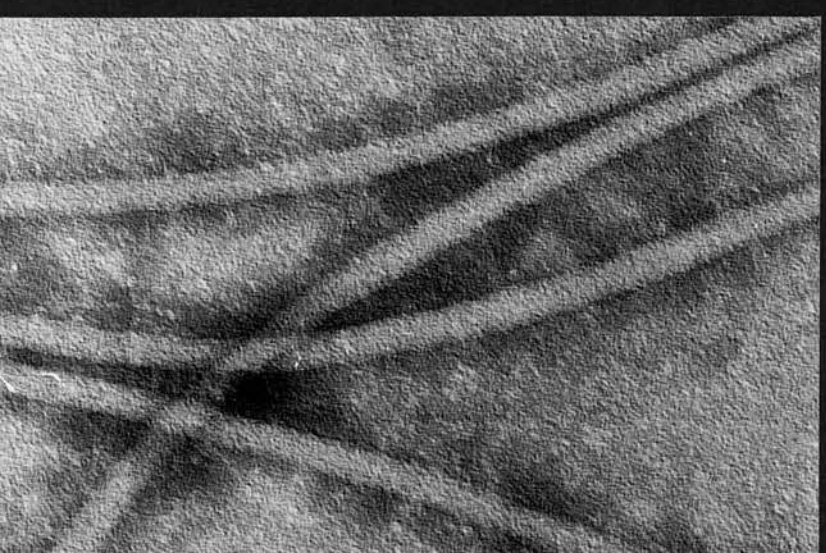


Fig. 4A

C. sputorum ss mucosalis obtained from a 24-hr-old culture treated with formaldehyde. Note the appearance and relative positions of the cytoplasmic membrane (CM), cell wall (CW) and rugose layer of the outer cell surface structures (OL). Some disruption of the outer layers by formaldehyde can also be seen (arrow). META X 69000.

Fig. 4B

A similar preparation to Fig. 4A showing the structure of the detached outer cell surface (arrow) which is approximately hexagonal in appearance. The contracted cytoplasmic mass and the 'naked' cell wall (CW) are also clearly seen. META X 90000.

Fig. 4C

Exposed cell wall of C. sputorum ss mucosalis after treatment with formaldehyde showing a number of unidentified approximately spherical structures. META X 187500.

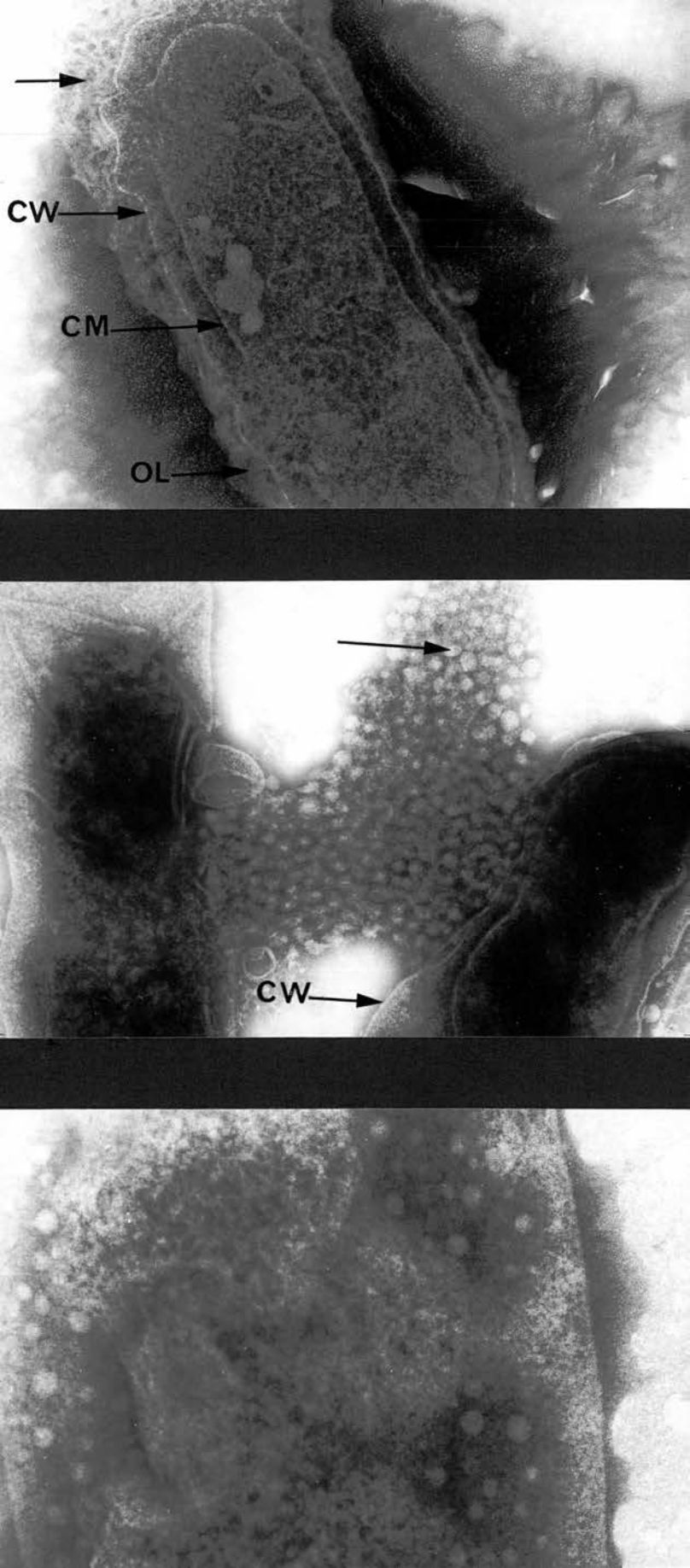

CW
CM
OL
CW

Fig. 5

Ultra-thin sections of C. sputorum ss mucosalis.

A. Note the double-layered cytoplasmic membrane (CM) and cell wall (CW). X 150000.

B. In this preparation the cytoplasmic contents are clearly seen. X 247500.

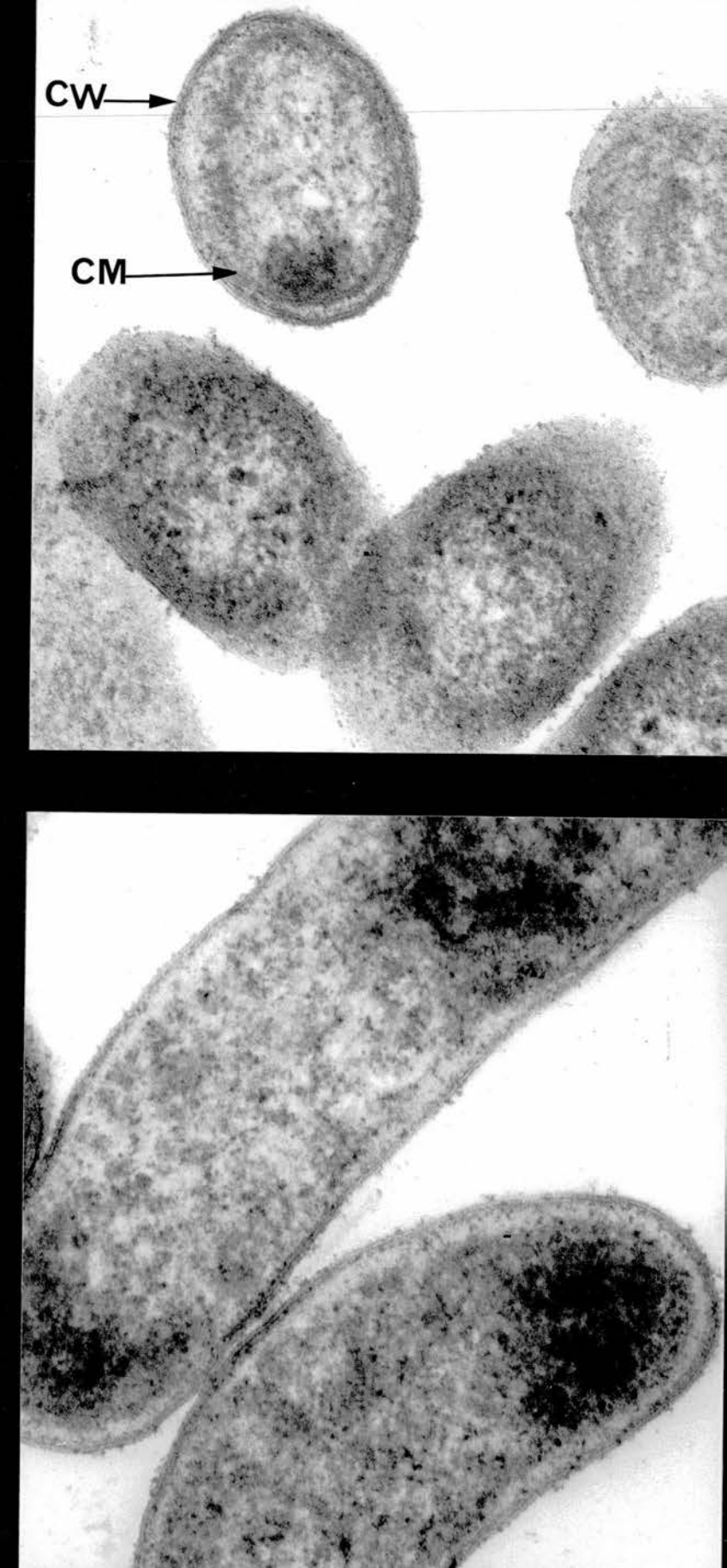

CW
CM

Fig. 6A

A negatively-stained preparation of C. coli strain A 124/73 A4. The cell surface appears to be 'leathery' with deep transverse clefts. One-day-old CBA culture stained with META X 52500.

Fig. 6B

In four-day-old CBA or CBA-MEM diphasic cultures of C. coli, the organisms may or may not have a leathery appearance. At this stage, coccoid forms are found. (arrow). META X 32250.

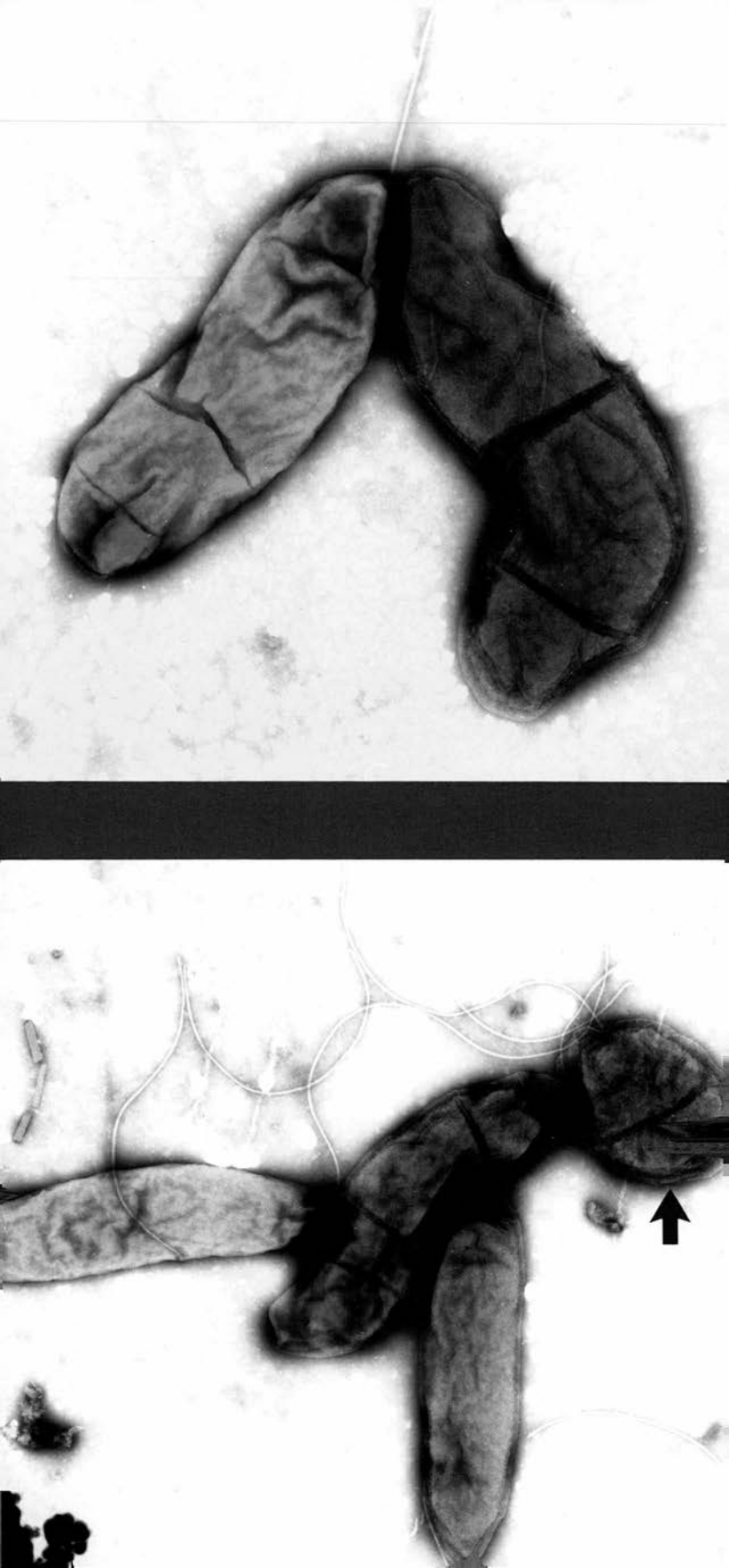
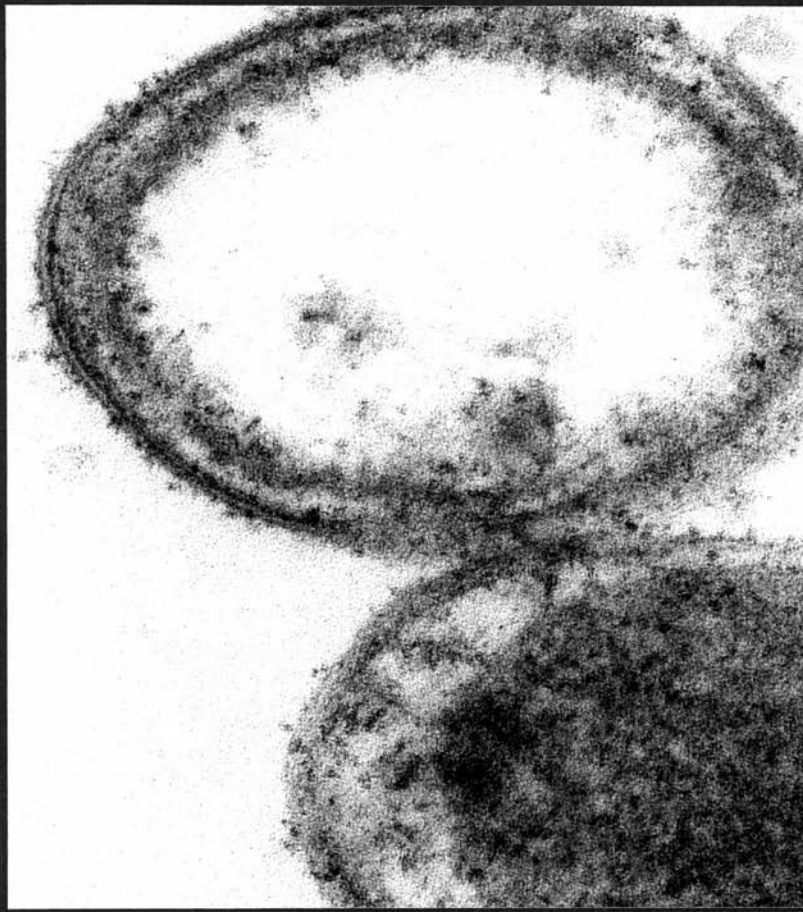


Fig. 7 Ultrathin sections of C. coli

A. Notice the large cytoplasmic vacuoles and aggregates of polyphosphate crystals. META X 115500.

B. The two coccoid forms clearly show the presence of double-layered cell walls and cytoplasmic membranes. META X 247500.



d. Comment.

Negatively-stained preparations show that all four strains of mucosalis are morphologically indistinguishable and show a similar cell surface topology which is probably dependent on the age of the culture and the nature of the growth medium. Moreover, the changes in the cell surfaces observed in this present study might be of considerable practical importance in evaluating the biological properties of the cell wall, since the significance of cell surface components in the attachment of pathogenic bacteria to host-cell surfaces has been emphasized by Rogers (1979) and Ward and Berkeley, (1980).

There appears to be some differences in the morphology and structure of the cell surfaces of mucosalis and C. coli organisms and it is of interest that mucosalis organisms invariably showed a single polar-flagellum compared with bipolar flagella in the strain of C. coli examined.

The ultrastructure of these two species of the genus Campylobacter was similar in that both possessed double-layered cell walls and membranes, and showed the presence of polyphosphate crystals. However the cytoplasmic 'vacuolar structure' was restricted to C. coli cultures.

It should also be mentioned at this stage that alteration of the methods of prefixation of bacterial cells followed by selective processing, provides more detailed information on the ultrastructure of bacteria (Ritchie et al., 1966; Rowles et al., 1976). In the present investigation, bacterial cells prefixed in glutaraldehyde were

routinely processed for thin-sectioning and therefore the possible existence of other bacterial structures (e.g. microcapsule) in mucosalis and C. coli strains cannot be fully discounted at least at this stage. Further detailed studies, particularly with isolated components of the bacterial cells, are necessary to provide a comprehensive picture of the ultrastructure of these organisms.

DISCUSSION.

This preliminary investigation of the morphology and ultrastructure of C. sputorum ss mucosalis and C. coli has been of considerable value and provides the basis for further studies on the relationships of these organisms with cell cultures.

The morphology of mucosalis organisms, in negatively-stained preparations, indicates a close resemblance to the comma and S-shaped but not coccoid forms of C. fetus ss fetus (Rhoades, 1954; Werner et al., 1961; Werner, 1963; Ritchie et al., 1966) and C. fetus ss jejuni (Pead, 1979). Although transverse clefts were observed on the cell surfaces of all three bacterial species (including mucosalis), further cell surface changes including the production of bacterial forms with longitudinal ridges and smooth cell surfaces have not been observed by the above workers either in C. fetus ss fetus or C. fetus ss jejuni. Also there appears to be considerable variation in the types of bacterial flagella, and mucosalis organisms, unlike C. coli, C. fetus ss fetus or C. fetus ss jejuni,

invariably possess a single polar flagellum. Polyphosphate crystals were commonly present in all these species of Campylobacter.

Ultrastructurally, mucosalis organisms resemble C. fetus ss fetus and C. coli although the presence of a trilamellar cell wall in C. fetus ss fetus and that of a microcapsule in C. fetus ss intestinalis have been suggested by Keeler et al., (1966) and Winter et al., (1978), respectively. As mentioned earlier, the techniques employed by these workers in the prefixation and subsequent processing of bacterial specimens were basically different from those used in the present investigation and for this reason the minor morphological differences described above required to be confirmed.

The demonstration of hexagonal cell surface structures obtained after formaldehyde treatment of C. sputorum ss mucosalis is of interest since McCoy et al., (1976) have shown that cell wall material of C. fetus ss intestinalis consisted of i) major components of loosely packed hexagonal subunits, and ii) minor components consisting of tightly packed hexagons. Similar hexagonal subunits have also been described on the surface of other bacteria (Murray, 1963; Kreig, 1976).

CHAPTER IV

CHAPTER IV

DEVELOPMENT OF CELL CULTURE INFECTION TECHNIQUES.

GENERAL INTRODUCTION.

C. sputorum ss mucosalis was first isolated from clinical cases of porcine intestinal adenomatosis (PIA) by Lawson and Rowland (1974), and subsequent studies by these workers have contributed greatly to our knowledge of this organism and its association with enteric disorders in swine.

Although bacteriological techniques have been successfully developed for the isolation and maintenance of this organism, cell culture systems have not been used previously to investigate mucosalis-host cell interactions. Unlike most pathogenic bacteria that have been studied in cell culture systems (Chapter I), mucosalis is unusual in that it does not grow under aerobic conditions and requires a hydrogen microaerophilic atmosphere for routine growth and maintenance. By contrast, animal cell cultures do not require such an atmosphere for satisfactory growth and it was doubtful if the hydrogen microaerophilic atmosphere would support the growth of cell cultures infected with mucosalis.

Most cell culture systems rely heavily on the action of antibiotics to maintain freedom from bacterial contamination. Such a procedure was largely ruled out, at least at this stage in the investigation, as clearly many antibiotics were likely to interfere with the growth and survival of mucosalis organisms. It was not known whether

contamination would present an insuperable problem to the investigation and it remained throughout a hazard which had to be constantly guarded against.

This chapter deals with the steps that were taken to develop a satisfactory cell culture infection procedure and describes the various stages in the growth of mucosalis in different types of cell cultures.

A. GROWTH OF C. SPUTORUM SS MUCOSALIS IN CELL CULTURE MEDIA.

a. Introduction.

C. sputorum ss mucosalis does not grow well in fluid bacteriological media even in the presence of a hydrogen microaerophilic atmosphere and has a marked preference for diphasic medium such as overlaid columbia blood agar (CBA). For this reason it was considered essential, before undertaking cell culture experiments, to study the ability of various types of cell culture media to support the growth of this organism and the following experiment was under taken to evaluate the growth of mucosalis in cell culture media in a hydrogen microaerophilic atmosphere.

b. Design of experiment.

Diphasic growth medium was prepared using an overlay of 20 ml of each of the following as the fluid phase over CBA: Dulbecco's MEM, MEM with tryptose phosphate broth (10%, TPB), Hanks' MEM and Medium 199, and all were supplemented with 10% heat inactivated calf serum.

Each of these modified diphasic media was inoculated with 2 ml of a 24 hr-old CBA diphasic growth of mucosalis strain 253/72 (approximately 6×10^7 bacteria/ml) and

incubated at 37°C under a hydrogen microaerophilic atmosphere, as described earlier (Chapter II). CBA diphasic media incorporating tryptose phosphate broth and 10% calf serum, inoculated and incubated as before, were used as controls. Bacterial counts were carried out at 24, 48 and 72 hr post-inoculation and the procedure followed was similar to that of the surface viable count method described by Miles et al., (1938). Serial 10-fold dilutions from each of the diphasic media were prepared in sterile PBS to give dilutions up to 10^{-9}. Using a dropper pipette, 0.02 ml of each dilution was deposited on the surface of two dry CBA plates, and after holding them for 10 min at room temperature, the inoculated plates were incubated at 37°C for 48 hr in a hydrogen microaerophilic atmosphere. The number of colonies of mucosalis in the highest dilutions yielding growth were counted, and the average number of colonies on two CBA plates was used to evaluate the rate of growth of mucosalis in different types of tissue culture media and in CBA/diphasic controls.

c. Results.

The data shown in Table 2 indicate that Dulbecco's MEM was superior to other tissue culture media. MEM supplemented with TPB proved to be unsatisfactory because the cultures showed marked turbidity with deposits. It was felt that these might interfere with the examination of cell cultures and might also indicate bacterial autoagglutination and instability. The other media used in this experiment did not produce turbidity or deposits, but Hanks'

TABLE 2

VIABLE COUNTS OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72 GROWN IN DIFFERENT TYPES OF CELL CULTURE MEDIA*.

Hours (h) or days (d) after inoculation.	Growth media				
	CBA+TPB	CBA+TPB+MEM (Dulbecco's)	CBA+MEM (Dulbecco's)	CBA+MEM (Hanks')	CBA+M199
0 h	6.78**	6.78	6.78	6.78	6.78
1 d	8.17	8.24	8.15	6.69	7.54
2 d	9.69	9.69	8.47	7.9	8.0
3 d	8.24	9.69	8.69	7.04	7.17

* = Incubated at 37°C under a hydrogen microaerophilic atmosphere.

** = Bacterial counts expressed as organisms $\log_{10}$/ml, corrected to the second decimal place.

MEM and Medium 199 supported only moderate growth of mucosalis.

B. EFFECT OF THE HYDROGEN MICROAEROPHILIC ATMOSPHERE ON THE GROWTH OF CELL CULTURES.

a. Introduction.

C. sputorum ss mucosalis is a fastidious microorganism which requires a hydrogen microaerophilic atmosphere for its growth and maintenance. In the absence of such a gaseous environment the bacterium is believed to be rapidly killed. Although the results obtained in the previous experiment show that MEM supported the growth of mucosalis when incubated under hydrogen microaerophilic atmosphere, it was considered necessary to study the response of cell cultures to this altered gaseous environment before undertaking in vitro infectivity experiments with this organism.

b. Design of experiment.

Initially PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cell lines were examined. In each case, one-week-old monolayer cultures were trypsinized with STV at 37°C and cell suspensions containing 10^5 cells per ml in MEM were seeded in 4oz medical flats. The flats, with loose caps, were then placed vertically in a McIntosh and Fildes' jar and the atmosphere replaced with hydrogen and carbon dioxide by the method described earlier for the growth of mucosalis (Chapter II).

Immediately after gassing, the anaerobic jars were opened and the caps of the flats were tightened and the bottles placed horizontally in the incubator at 37°C for

1 week. Cells were neither regassed nor refed with fresh medium during the period of the experiment. As controls, monolayers were grown in medical flats at 37°C without gassing. In every case the ability of cells to grow, attach to the surface of the glass and form confluent monolayers was assessed daily by light microscopy.

c. Results.

It was noted in all cell lines examined that the hydrogen microaerophilic atmosphere did not affect cell attachment and subsequent growth during the first 48 hours of incubation. However, after the second day, large numbers of cells were seen to be "rounding up" and becoming detached from the surface of the glass until, by the end of the 4th day, very little of the monolayer remained. This indicated that altered gaseous environment was not conducive to the growth and survival of any of the cell lines examined.

Additional experiments involving 1 to 2 day-old preformed monolayers of PK, PK_{pi}, BK and BK_{pi} and OK_{pi} cells grown in 4 oz medical flats, inoculated with 10 ml of MEM supplemented with 2 ml of a 24 hr-old diphasic growth of mucosalis strain 253/72 and incubated in a hydrogen microaerophilic atmosphere, showed rapid destruction of the infected monolayers in less than 3 days.

d. Comment.

The results of this experiment clearly show that incubation of cell cultures, whether infected with mucosalis or not, in a hydrogen microaerophilic atmosphere is detrimental to the growth of cells, and that the procedure is

likely to be unsatisfactory for long term studies of mucosalis-host cell relationships.

C. GROWTH OF C. SPUTORUM SS MUCOSALIS IN CELL CULTURES INCUBATED UNDER AEROBIC CONDITIONS.

a. Introduction.

In view of the difficulties experienced in the growth and maintenance of cell cultures under a hydrogen microaerophilic atmosphere, an attempt was made to ascertain whether cell cultures are capable of supporting the growth of C. sputorum ss mucosalis in the absence of a hydrogen gaseous environment. The following experiment was designed to study the growth of this organism in cell cultures incubated under conventional 'aerobic' conditions.

b. Design of experiment.

Confluent monolayers of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cells grown for 1 to 2 days in medical flats were overlaid with 10 ml of a bacterial suspension in prewarmed ($37^{o}C$) MEM containing 5% heat inactivated calf serum. Approximately 2×10^{7} bacteria/ml obtained from a 24 hr CBA-MEM diphasic growth, were used to infect the cell cultures. All cell cultures, both uninoculated and mucosalis-inoculated monolayers were incubated at $37^{o}C$ without added hydrogen and carbon dioxide. The viability of mucosalis was checked by plating 0.2 ml of the supernatant medium on two dry CBA plates and, after holding them for 10 min at room temperature, the inoculated plates were incubated at $37^{o}C$ in a hydrogen microaerophilic atmosphere. This procedure was repeated daily for one week without refeeding

TABLE 3

ISOLATION OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72

FROM INFECTED CELL CULTURES.

Days (d) or weeks (w) after inoculation.	Cell types examined				
	PK	PK_{pi}	BK	BK_{pi}	OK_{pi}
1 d	+++	+++	+++	+++	+++
2 d	+++	+++	+++	+++	+++
3 d	+++	+++	+++	+++	+++
4 d	++	++	+++	+++	+++
5 d	++	++	+++	+++	+++
1 w	-	-	+++	+++	+++
2 w	-	-	+++	+++	+++
3 w	-	-	+++	+++	+++

+++ = Confluent bacterial surface growth with 0.2 ml of inoculum.

++ = Less than confluent surface growth with 0.2 ml of inoculum.

- = No bacterial growth.

Similar results were obtained after infection of suspensions of trypsinized cells or preformed monolayers.

the monolayer cultures. The plates were examined for the presence of mucosalis colonies after incubation for 48 hr.

c. Results.

Examination of CBA plates inoculated with supernatant tissue culture fluids obtained from all the infected cell lines showed confluent growth of mucosalis organisms. It is evident from the data shown in Table 3 that it was possible to isolate bacteria from PK, and PK_{pi} cells for only 5 days whilst other cell lines yielded bacteria till the end of this experiment.

d. Comment.

The ability of mucosalis to grow in cell cultures in the absence of a hydrogen microaerophilic atmosphere was an unexpected observation which is clearly of the utmost importance for future studies on the relationship of this organism with cultured cells.

D. MODIFICATIONS IN CELL CULTURE INFECTION PROCEDURES.

a. Introduction.

When the cell cultures were exposed to infection with C. sputorum ss mucosalis in a hydrogen microaerophilic atmosphere, death of the cells took place within 3 days. Whilst it seemed likely that this was a direct consequence of bacterial multiplication in the fluid medium it was also possible that some of the degenerative changes were due to the presence of bacterial metabolites in the inoculum. In order to minimise such a possibility, the mucosalis inoculum was modified as follows.

b. Design of experiment.

i. Preparation of bacterial inoculum.

A 24 hour-old surface growth of mucosalis strain 253/72 on a CBA slope was removed by gentle washing with 10 ml of prewarmed (37°C) MEM. The suspension of bacteria thus obtained generally contained approximately 1-2 x 10^7 organisms/ml as indicated by surface viable counts (Miles et al., 1938).

ii. Infection of preformed monolayers with mucosalis.

Confluent monolayers of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cells grown for 24 hr in 4 oz medical flats were overlaid with 10 ml of a suspension of mucosalis in MEM and incubated at 37°C in 'aerobic' conditions. Each day during the first week and thereafter at weekly intervals for 3 weeks, 0.2 ml of the supernatant fluid from each infected cell culture was uniformly spread on two dry CBA plates. The plates were then held at room temperature for 10 to 15 min prior to incubation for 48 hr at 37°C under a hydrogen microaerophilic atmosphere and were then examined for the presence of bacterial growth.

c. Results.

The results were identical to those obtained with organisms derived from CBA-MEM diphasic growth of mucosalis and are shown in Table 3.

d. Comment.

Since the results obtained with surface growth cultures of mucosalis were similar to those with diphasic cultures, there appeared to be no particular advantage in using the more complex diphasic procedure as a source of bacterial

inoculum. For this reason and because this method of infecting cell cultures should reduce the toxic effects of bacterial metabolites on cell cultures, the surface growth of mucosalis obtained from 24 hr-old CBA slope cultures was routinely used in all further experiments unless stated otherwise.

E. THE GROWTH OF C. SPUTORUM SS MUCOSALIS IN TRYPSINISED CELL SUSPENSION PRIOR TO MONOLAYER FORMATION.

a. Introduction.

Although preformed monolayers of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cultures supported the growth of C. sputorum ss mucosalis in the absence of a hydrogen microaerophilic atmosphere, it was necessary to establish if a similar type of 'parasitic growth' could be obtained by inoculating suspensions of trypsinized cells with mucosalis before the monolayers had formed. Such a procedure is also likely to provide additional information on the ability of different types of infected cultures to attach to the glass and form confluent monolayers.

b. Design of experiment.

i. Preparation of bacterial suspensions.

Bacterial suspensions from overnight cultures of mucosalis strain 253/72 were prepared by the procedures described in the previous experiment.

ii. Infection of trypsinized cells with mucosalis.

One-week-old monolayers of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cells were trypsinized with STV solution, and 10^6 cells

were immediately mixed with 10 ml of a suspension of mucosalis in MEM and allowed to form monolayers in 4 oz medical flats at 37°C under aerobic conditions. As controls, appropriate uninoculated trypsinized cells were allowed to form monolayers at 37°C.

iii. Infection of preformed monolayers with mucosalis.

Confluent monolayers of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cells grown for 24 hr in 4 oz medical flats were infected with mucosalis as described in the previous experiment and incubated at 37°C. Appropriate uninoculated cultures were used as controls.

iv. Examination of cell cultures infected with mucosalis.

Trypsinized cells infected with mucosalis organisms were routinely examined by light microscopy for their ability to attach to the glass and form confluent monolayers, and the results were compared with the uninfected controls. Infected trypsinized and preformed monolayer cultures were also examined for cytopathic abnormalities and pH changes, and the clarity of the supernatant medium was compared with that of uninfected controls.

The isolation of mucosalis organisms from the supernatant fluids of infected cultures was undertaken as described in the previous experiment.

c. Results.

Microscopic examination of unstained monolayers observed during the first 48 hr after infection showed no appreciable difference in the ability of infected trypsinized cells to attach to the glass or form confluent

monolayers compared with those of uninfected control cultures. However, by the 4th day, the monolayers obtained from infected trypsinized cells appeared to be less confluent compared with the cell sheets of the uninfected controls. Mucosalis infection did not appear to change the pH or the clarity of the supernatant medium and, in fact, with the unaided eye it was difficult to differentiate infected cultures from the controls.

It was interesting to note that all types of cell cultures infected immediately after trypsinization were capable of supporting the growth and multiplication of mucosalis in the absence of a hydrogen microaerophilic atmosphere. Mucosalis was isolated readily from infected trypsinized cell cultures and their recovery was similar to that observed in preformed monolayers infected with this organism (Table 3).

d. Comment.

The fact that trypsinized cells infected with mucosalis form confluent monolayers and support the growth of the organism may provide a useful alternative method for studying the behaviour of mucosalis in cell cultures. One of the advantages of this procedure is that the inoculum is added to the cells when they are actively dividing and are possibly more susceptible to infection.

F. THE ABILITY OF OTHER TYPES OF CELLS TO SUPPORT 'AEROBIC' GROWTH OF C. SPUTORUM SS MUCOSALIS.

a. Introduction.

Preliminary observations indicated that PK, PK_{pi}, BK,

BK_{pi} and OK_{pi} cell lines supported the growth and multiplication of mucosalis in the absence of a conventional hydrogen microaerophilic atmosphere. Other cell types derived from different species of animals were examined for their ability to support the 'aerobic' growth of mucosalis, and these included primary pig kidney cells (PPK) and chicken embryo fibroblasts (CEF).

b. Design of experiment.

In this experiment, BHK, DK, HeLa, Vero, LLCMK2, secondary CEF and PPK were exposed to infection with mucosalis strain 253/72. The preparation of the bacterial inoculum, cell infection procedures (including cultivation with trypsinized cells and one-day-old preformed monolayers) and routine light microscopy, as well as mucosalis isolation procedures, were similar to those described in the previous experiment (page. 96). Corresponding uninfected controls were also examined.

c. Results.

The data obtained from these experiments are summarized in Table 4. With the exception of Vero, LLCMK2 and CEF cells, all other cell types were capable of supporting the growth of mucosalis in the absence of a hydrogen microaerophilic atmosphere. The duration of persistence of bacteria in such 'permissive'cells appeared to be about 5 days or less in PPK cells whereas other cell cultures yielded bacteria for up to 3 weeks although the numbers recovered gradually decreased. Additional experiments conducted on Vero, LLCMK2 and CEF cells confirmed their

TABLE 4

ISOLATION OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72

FROM CELL CULTURES.

Days (d) or weeks (w) after inoculation	Cell types examined						
	PPK	CEF	BHK	Vero	$LLCMK_2$	DK	HeLa
1 d	+++	-	+++	-	-	+++	+++
2 d	+++	-	+++	-	-	+++	+++
3 d	+++	-	+++	-	-	+++	+++
4 d	++	-	+++	-	-	+++	+++
1 w	-	-	+++	-	-	+++	+++
2 w	-	-	+++	-	-	+++	+++
3 w	-	-	+++	-	-	+++	+++

+++ = Confluent bacterial surface growth with 0.2 ml of inoculum.

++ = Less than confluent bacterial surface growth with 0.2 ml of inoculum.

- = No bacterial growth.

Similar results were obtained after infection of suspensions of trypsinized cells or preformed monolayers.

inability to support the growth of mucosalis under aerobic conditions.

Certain cellular changes were observed in most types of permissive cells and these will be dealt with in detail at a later stage (Chapter VIII).

G. EFFECTS OF RE-INFECTION OF VERO, LLCMK2 AND CEF CELLS WITH C. SPUTORUM SS MUCOSALIS UNDER AEROBIC CONDITIONS.

a. Introduction.

Several attempts to demonstrate the 'aerobic' growth of C. sputorum ss mucosalis in cultured Vero, LLCMK2 and CEF cells were unsuccessful and this intriguing observation, which contrasted with the recovery of organisms from other types of permissive cells, merited further detailed investigations. In the following experiment an attempt was made to discover if this bacteria - cell relationship could be influenced by repeated infection of these cell lines with mucosalis organisms.

b. Design of experiment.

The methods of inoculation, incubation and isolation of mucosalis from infected trypsinized cell suspensions and preformed monolayers of Vero, LLCMK2 (one-day-old) and CEF (3 to 4 day-old) grown in 4 oz medical flats have been described earlier (page. 98). The cell cultures which had been exposed to infection were thoroughly rinsed in several changes of PBS to remove killed bacteria and then overlaid with 10 ml of a further 24 hr old suspension of mucosalis (approximately 1×10^7 bacteria/ml) in prewarmed MEM containing 5% calf serum.

Reinfected and 'once-exposed' control monolayers of each cell type were examined daily for 5 days for the growth of mucosalis by plating 0.2 ml of the supernatant tissue culture fluids on two dry CBA plates, incubated in a hydrogen-microaerophilic atmosphere. The ability of mucosalis to persist for longer periods in these re-infected cultures was also similarly examined at weekly intervals. The cytopathic changes observed by light microscopy are described in Chapter VIII.

c. Results.

Following re-infection of 'once-exposed' Vero and $LLCMK_2$ cells, viable mucosalis organisms were recovered for up to 2 weeks whereas 'once-exposed' control cultures remained negative during this period. These observations were confirmed using a second strain of Vero cells obtained from the Moredun Institute, Edinburgh.

By contrast, CEF cells failed to support the growth of mucosalis and attempts to isolate this organism as early as 24 hr after re-infection of 'once-exposed' cultures proved unsuccessful.

d. Comment.

The fact that growth of mucosalis in Vero and LLCMK2 cells could only be induced by reinfecting the previously exposed cultures is of interest although, unfortunately, no explanation can be given for this phenomenon. Moreover, it is emphasised that the same procedures failed to produce growth of mucosalis in CEF cultures.

H. SURVIVAL OF C. SPUTORUM SS MUCOSALIS IN CELL-FREE MEM UNDER AEROBIC CONDITIONS.

a. Introduction.

A quantitative assessment of the growth of C. sputorum ss mucosalis in CBA-MEM diphasic medium incubated in a conventional hydrogen microaerophilic atmosphere formed the basis of an experiment described earlier in this chapter. The ability of mucosalis to grow in different types of cell cultures maintained in MEM in the absence of such an atmosphere was intriguing and prompted further investigation. Thus the following experiment was designed to determine the ability of this organism to persist in cell-free MEM under 'aerobic' conditions.

b. Design of experiment.

A suspension of mucosalis strain 253/72 in prewarmed MEM was prepared as described earlier in this chapter. Two medical flats, each containing 10 ml of the bacterial suspension (approximately 1×10^7 bacteria/ml), were incubated horizontally at 37°C under 'aerobic' conditions, and each was examined at hourly intervals up to 12 hr and at 24 hr for the presence of viable organisms as follows. A small drop (0.1 ml) of infected MEM was spread uniformly on each of two dry CBA plates and the inoculated plates were then held at room temperature for 10-15 min prior to incubation at 37°C in a hydrogen microaerophilic atmosphere. After 48 hr incubation, the plates were examined for the presence of mucosalis colonies.

Additional experiments were carried out with other

strains of mucosalis (982/76; 512/77; 1075/78) and a strain of C. coli (124/73 A4) in order to compare their persistence in cell-free MEM incubated under 'aerobic' conditions.

c. Results.

Examination of the recovery of the strains of mucosalis clearly showed a rapid decrease in viable bacteria after 6 hr and no organisms could be recovered after 12 hr post-inoculation. This indicated total destruction of these bacteria in cell-free media under aerobic conditions.

In contrast, the strain of C. coli remained viable for up to 24 hr post-inoculation. In separate extended experiments viable C. coli organisms could be isolated for up to 4 days under these conditions.

d. Comment.

The results clearly indicated that mucosalis was rapidly killed when incubated at 37°C in cell-free MEM under aerobic conditions. Particular attention is drawn to the fact that the inoculation and incubation procedures of the suspension of mucosalis in this experiment were similar to those described previously for the infection of cell cultures. In the latter situation mucosalis remained viable and could be recovered for at least three weeks from some cell lines. This shows that cell cultures may support the growth and possible multiplication of mucosalis in the absence of a hydrogen microaerophilic atmosphere.

DISCUSSION.

The results of these experiments are of interest in so far as they identify some of the unusual features in the relationship between mucosalis organisms and infected cell cultures. Although the precise mechanisms involved in the 'parasitic multiplication' of mucosalis are not known, the evidence seems to indicate that they are closely associated with the survival of the infected cells. To a large extent, the viability of cell cultures depends on the type of medium used and the various metabolic and environmental factors which are necessary for cell growth. The gaseous atmosphere is also of importance and there is general agreement that both oxygen and carbon dioxide are essential for cell survival. Most systems require adequate carbon dioxide tension in the gaseous phase and in the medium, and this is usually achieved by growing the cells in sealed culture vessels.

In our experiments with mucosalis, the metabolism of actively growing cell cultures and retention of carbon dioxide in tightly sealed containers seemed to simulate the hydrogen microaerophilic atmosphere which is essential for the growth of this organism.

'Parasitic' multiplication of mucosalis appeared to be selective and, in PK, PK_{pi} cells at least, bacterial viability is probably related to the survival of host cells since the organisms rapidly die when cellular destruction is extensive. In other cell systems, the effects of mucosalis seemed to depend on the type of cell

employed. For example, in BK, BK_{pi}, OK_{pi}, BHK, DK and HeLa cell lines the infected cells remained attached to the glass and continued to support bacterial growth for several weeks whereas CEF, Vero and $LLCMK_2$ cells appeared to be refractory to the infection and the organisms rapidly disappeared from the intact monolayer cultures. Although repeated inoculation of Vero and $LLCMK_2$ cell lines with mucosalis induced 'parasitic' growth, similar attempts to induce the infection in secondary cultures of CEF proved unsuccessful.

Other organisms which are normally CO_2-dependant are also capable of growth in cell culture systems. For example, a strain of C. coli (124/73 A4) which is less fastidious than mucosalis grew well in all types of cell cultures employed in this present study including CEF, Vero and $LLCMK_2$ cells. However, it should be emphasized that this organism, unlike mucosalis, was also capable of survival in cell-free MEM upto 4 days post-inoculation.

Little comparative information is available on 'parasitic' bacterial growth, other than for obligate intracellular parasites; but a recent report (Sandok et al., 1978) has shown that the extracellular parasite, Treponema pallidum, appeared to grow better in cell culture systems (5 days) than in cell-free tissue culture media (4 days).

CHAPTER V

CHAPTER V

BACTERIOLOGICAL INVESTIGATIONS OF CELL CULTURES INFECTED WITH C. SPUTORUM SS MUCOSALIS.

GENERAL INTRODUCTION.

Numerous in vitro studies have been made on the association of pathogenic bacteria with cell cultures, and it is apparent from published findings on the nature of bacterial-host cell interactions that an increase in the number of 'cell-associated' organisms has been generally accepted as evidence of intracellular multiplication. There has, however, been a lack of uniformity in the techniques employed both in experimental cell infection and in bacterial recovery. The extent of intracellular growth has been assessed in a variety of ways, on the basis of bacterial counts in stained preparations, viable counts of the infecting organism and, occasionally, by immunofluorescence.

The counting of bacteria in stained preparations of infected cell cultures does not appear to be a completely satisfactory procedure as it fails to distinguish intracellular bacteria from those located on the cell surfaces. However, this technique has been of value in evaluating the intracellular growth of a number of fastidious microorganisms such as mouse leprosy bacillus (M. lepraemurium) which does not grow on conventional bacteriological media and the growth of which can only be quantified by counting the number of stained organisms in cell cultures.

Assessment of bacterial proliferation in infected tissue culture cells by the viable bacteria that can be recovered from such systems clearly appears to be a more accurate method of assaying bacterial growth. Since only live organisms are recovered, the information obtained is not confused by the presence of phagocytosed or dead bacteria. Despite this advantage there are many problems, not the least being extracellular multiplication in the fluid phase of cell culture systems. Many workers have attempted to overcome this problem by including antibiotics in cell culture media, although the procedure is likely to introduce new problems in the interpretation of results. The extent to which antibiotics are excluded from the cell cytoplasm is not always clear and their presence may interfere with the intracellular survival and multiplication of bacteria. In addition, bacteria may develop 'resistant forms' against the antibiotics in question and these might continue to multiply within or outwith the cell in the presence of antibiotic substances.

Immunofluorescence staining has been used by several workers for the assessment of intracellular bacterial multiplication. This technique however has similar drawbacks to light microscopy of stained preparations in that it often fails to distinguish clearly between organisms located within the cell or on the cell surfaces. In addition it readily demonstrates dead bacteria and bacteria-derived intracellular particulate antigens which

may, therefore, be confused with live organisms.

Of the methods employed to quantify intracellular multiplication of bacteria, the technique of viable counting of organisms in the infected cell cultures is not without problems of interpretation but appears to be capable of yielding useful information not provided by other methods.

This chapter deals with attempts to quantify and investigate more fully the 'parasitic' growth of mucosalis in cell culture systems. The terms 'parasitic growth' or 'parasitic multiplication' will be used to describe the continued presence of viable mucosalis organisms in association with cultured cells grown under conventional 'aerobic' atmosphere, without implying any knowledge of the relationship which permits this prolonged growth and survival of the organism.

I. PERSISTENCE OF 'PARASITIC GROWTH' OF C. SPUTORUM SS MUCOSALIS IN CELL CULTURES.

a. Introduction.

Preliminary investigations have suggested that cell cultures vary in their ability to support 'parasitic growth' of C. sputorum ss mucosalis under similar experimental conditions. For example, PK, PK_{pi}, and PPK cells produced 'parasitic growth' for only 5 days compared with BK, BK_{pi}, OK_{pi}, BHK, HeLa and DK cells which yielded the growth of bacteria during the whole three week period of the experiment (Chapter IV, pp.93, 100).

In the above experiment, which was basically a preliminary attempt to grow mucosalis under 'aerobic' conditions, the infected cell cultures had not been refed and this might have allowed extracellular bacteria to persist for longer periods and also prove detrimental to cell cultures. It was therefore necessary to examine the effects of regular refeeding of infected cell cultures on the persistence of 'parasitic growth' of mucosalis, and the following experiment was carried out to seek an answer to this question.

b. Design of experiment.

Several monolayers of BK, BK_{pi}, OK_{pi}, BHK, HeLa and DK cells, grown overnight in 4 oz medical flats, were separately overlaid with 10 ml of a bacterial suspension of each of the mucosalis strains, 253/72, 982/76, 512/77 and 1075/78. The bacterial inoculum contained approximately 2×10^7 organism/ml and was obtained from appropriate 24 hr-old surface growths on CBA slopes (Chapter IV, p. 95).

The 'parasitic growth' of mucosalis strains in these cultured cells was examined at weekly intervals by uniformly spreading 0.2 ml of the supernatant fluids obtained from each medical flat on each of two dry CBA plates. The inoculated plates were then held at room temperature for 10-15 min prior to incubation under a hydrogen microaerophilic atmosphere and were examined for the presence of mucosalis colonies after 24 and 48 hr.

After sampling the supernatant fluids for the isolation of mucosalis, the infected monolayers were refed with 10 ml of prewarmed (37°C) MEM containing 5% calf serum and reincubated at 37°C. These sampling and refeeding procedures were repeated at weekly intervals till the cultures proved negative for mucosalis on two successive occasions.

In parallel experiments, trypsinized cells of BK, BK_{pi}, OK_{pi}, HeLa, BHK and CK cultures, separately mixed with 10 ml of a suspension of each mucosalis strain 253/72, 982/76, 512/77 and 1075/78, were allowed to form monolayers in 4 oz medical flats as described earlier (p.96). These were examined culturally for the isolation of mucosalis and refed at weekly intervals as described above.

In all cases, corresponding uninfected monolayers were routinely examined by light microscopy.

c. Results.

The data presented in Table 5 indicated that different types of cell cultures showed some variation in their ability to support sustained 'parasitic growth' of mucosalis organisms. All the four strains of mucosalis examined in this experiment behaved similarly and were recoverable in sufficient numbers upto 6 to 7 weeks in BK cells and for slightly shorter periods (5-6 weeks) in other cell types.

Identical observations were also made in suspensions of trypsinized cells of BK, BK_{pi}, and OK_{pi} lines infected

TABLE 5

LONG TERM RECOVERY OF *C. SPUTORUM* SS *MUCOSALIS* FROM INFECTED CELL CULTURES.

Weeks after inoculation	Cell types examined					
	BK	BK_{pi}	OK_{pi}	BHK	DK	HeLa
1	+++	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++	+++
4	+++	+++	+++	++	++	++
5	++	++	++	a	a	a
6	++	a	a	-	a	a
7	a	-	-	-	-	-
8	-	-	-	-	-	-

+++ = Confluent bacteria surface growth with 0.2 ml of inoculum.

++ = Less than confluent bacterial surface growth with 0.2 ml inoculum.

a = A few colonies of *mucosalis* invariably present. - = No bacterial growth.

Similar results were obtained with *mucosalis* strains 253/72, 982/76, 512/77 and 1075/78.

with mucosalis strains and these results indicated that modifications in cell culture infection procedures did not influence the persistence of 'parasitic growth'.

Microscopical examination of infected monolayers confirmed that failure to isolate mucosalis was associated with total destruction of cell sheets in BHK and HeLa cultures but not with other cell types.

The colonial morphology of mucosalis strains isolated from the infected cell cultures was similar to that of mucosalis grown on bacteriological media under a hydrogen microaerophilic atmosphere.

d. Comment.

The persistence of 'parasitic growth' of mucosalis over a period of 5 to 7 weeks in the presence of cultured BK, BK_{pi}, OK_{pi}, BHK, DK and HeLa cells was an important observation and clearly suggests that:

i. a sustained multiplication of mucosalis has taken place which appears to be dependant on the survival of the host cells, at least in some cell types.

ii. unlike most pathogenic bacteria that have been studied in cell cultures, mucosalis does not rapidly destroy the infected cells. Certain types of cell lines, particularly BK cells, appear to withstand the presence of mucosalis organisms over prolonged periods and are less susceptible to the destructive changes due to infection with this organism.

iii. 'parasitic growth' in cell cultures does not induce any evidence of 'phase variation' in mucosalis

organisms as demonstrated by alterations in colonial morphology although this has often been observed with Brucella species and N. gonorrhoeae grown in cell culture systems (Egwu and Eveland, 1979; Gavrilescu et al., 1966).

The nature of the unusual 'parasitic growth' of mucosalis is not fully known, and further investigations are therefore necessary to ascertain whether or not this 'parasitic growth' is due to intracellular or cell-dependant extracellular multiplication.

II. A QUANTITATIVE ASSESSMENT OF 'PARASITIC GROWTH' OF C. SPUTORUM SS MUCOSALIS IN CELL CULTURES.

a. Introduction.

The 'parasitic growth' of C. sputorum ss mucosalis observed in different types of cell cultures was an interesting, and largely unexpected observation. In this context it will be recalled that although mucosalis is not generally regarded as an obligate intracellular parasite, there is evidence from immunofluorescence and electron microscopic studies (Rowland and Lawson, 1974; Roberts, 1978) of its intracellular location and apparent multiplication in adenomatous intestinal epithelial cells of affected pigs.

With this in mind, an attempt was made to ascertain whether or not 'parasitic growth' or mucosalis is the product of intracellular bacterial multiplication or is

merely a cell-associated or cell-dependant type of extracellular bacterial growth. This information is critical to the assessment of mucosalis infection of cell culture systems and may have possible implications in analysing the disease process in the pig.

The first step is to assess quantitatively the extracellular bacterial growth present in the supernatant cell culture fluids since this would indicate whether mucosalis multiplies in infected cell cultures or merely persists at the level at which the organism was inoculated. Additionally, such information will serve as a base line for the assessment of intracellular bacteria after the extracellular organisms have been eliminated by various treatments. The following experiment was designed to evaluate the concentration of extracellular mucosalis in infected cell cultures.

b. Design of experiment.

One-day-old monolayers of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK and HeLa cells,grown in 4 oz medical flats, were overlaid with 10 ml of a suspension of mucosalis strain 253/72 in MEM and incubated at 37°C. The bacterial inoculum was obtained by the procedure described earlier (Chapter IV, p.95) and the number of organisms present was determined by the surface viable counting technique described on p. 88. Bacterial counts were also carried out on the supernatant fluids of two infected monolayers of each cell type daily during the first 5 days, and

then at weekly intervals for 8 weeks post-inoculation.

The inoculated CBA plates were held at room temperature for 10 to 15 min prior to incubation (37°C) in a hydrogen microaerophilic atmosphere. After 48 hr the number of colonies of mucosalis on each plate were counted and an average of the two corresponding dilutions was used to calculate the number of bacteria present in the supernatant fluids. The infected monolayers were not refed during this period.

c. Results.

The viable counts of mucosalis as presented in Table 6 clearly illustrate the rapid disappearance of a large proportion of the inoculated bacteria within the first 24 hr post-inoculation in all cell types examined. After this initial drop the number of mucosalis organisms steadily increased and reached a maximum around 4 to 5 days post-inoculation. However, this 'parasitic growth' failed to achieve the concentration of bacteria originally present in the inoculum.

Statistical analysis of the bacterial counts on days 1 and 5, irrespective of the cell type involved, indicated that the increase was highly significant ($t = 6.93$ significant at <0.01). These results also confirmed earlier observations that BK, BK_{pi}, and OK_{pi} cells produced 'parasitic growth' over prolonged periods (5 to 7 weeks).

TABLE 6

NUMBERS OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72 IN THE SUPERNATANT FLUIDS OF INFECTED CELL CULTURES.

Hours (h), days (d) or weeks (w) after inoculation.	Cell types examined							Control
	PK	PK_{pi}	BK	BK_{pi}	OK_{pi}	BHK	HeLa	Cell-free MEM Aerobic incubation
0h	6.88*	6.88	6.88	6.88	6.88	6.88	6.88	6.88
1d	3.30	3.18	2.88	3.00	3.10	3.18	3.30	-
2d	3.70	4.00	3.18	3.24	3.30	3.40	3.54	-
3d	4.18	4.30	3.60	3.70	3.66	3.54	3.65	-
4d	4.60	4.51	4.48	4.30	4.40	4.40	4.17	-
5d	5.00	5.30	4.65	3.60	4.63	4.40	4.48	NT
1w	-	-	4.70	3.35	3.30	3.30	3.60	NT
2w	-	-	3.0	3.0	3.18	3.18	3.10	NT
3w	-	-	a	a	a	a	a	NT
4w	-	-	a	a	a	a	a	NT
5w	-	-	a	a	a	a	a	NT
6w	-	-	a	a	a	-	a	NT
7w	-	-	a	-	-	-	-	NT
8w	-	-	-	-	-	-	-	NT

* = Bacterial counts expressed as organisms $\log_{10}$/ml, corrected to the second decimal place.

a = A few colonies of mucosalis invariably present.

\- = No bacterial growth.

NT = Not tested.

d. Comment.

The earlier observation that C. sputorum ss mucosalis is killed in about 12 hr in cell-free MEM and the present finding that the majority of bacteria are also rapidly destroyed within 24 hr in cultured cells suggest that the factors responsible are the same or very similar, and that the sharp decline in the numbers of viable organisms is partly associated with phagocytosis and extracellular death.

The survival of a small number of bacteria in the presence of cultured cells indicated that the cultures are only capable of supporting limited but sustained multiplication of a small proportion of inoculated bacteria. This observation infers that the favourable conditions produced by the metabolism and active growth of cells is not solely responsible for the parasitic growth of mucosalis, otherwise larger numbers of bacteria might be expected to survive. Therefore, in some way, the 'parasitic growth' of mucosalis appears to depend more on its association with the host cells than on the gaseous atmosphere produced by the cell cultures. Further studies are required to explain this complex mucosalis-host cell relationship.

In view of these facts the prolonged persistence of mucosalis in certain types of cell cultures is intriguing and may not be entirely due to cell-dependant extracellular growth of the organism. Until suitable experimental procedures are developed for distinguishing

between intra- and extracellular growth of the organism, it will be difficult to identify the origins of 'parasitic' bacteria. Further investigations are required to clarify this issue.

III. DEVELOPMENT OF TECHNIQUES FOR THE ELIMINATION OF EXTRACELLULAR 'PARASITIC GROWTH' OF C. SPUTORUM SS MUCOSALIS.

a. Introduction.

Several workers have attempted to demonstrate intracellular multiplication of pathogenic bacteria in cell cultures and have found that the incorporation of appropriate antibiotics in the cell culture media is probably the most useful means of eliminating extracellular organisms (Holland and Pickett, 1956; Richardson and Holt, 1962; Hatten and Sulkin, 1966a, b; Kihlstrom, 1977; Egwu and Eveland, 1979; Brunius, 1980). The literature on this subject has been briefly reviewed in Chapter I and it is evident that a great deal of uncertainty exists as to the concentration, duration and types of antibiotics necessary to achieve this objective.

The first step in the use of antibiotics to prevent the extracellular growth of mucosalis strain 253/72 in cell cultures, was to examine a wide range of antibiotics for their ability to inhibit this organism. The following experiment was designed for this purpose.

A. DETERMINATION OF ANTIBIOTIC SENSITIVITY OF C. SPUTORUM SS MUCOSALIS.

b. Design of experiment.

In principle, the technique used was similar to that described by Cruickshank (1965). Briefly, a 24 hr-old CBA growth of mucosalis strain 253/72 was suspended in tryptose phosphate broth and uniformly spread on dry CBA plates. Plates inoculated in this way were then held at room temperature for 10-15 min before being overlaid separately with 'Oxoid multodisks' (Oxoid, London) impregnated with a number of antibiotics: penicillin (1.5 IU), ampicillin (2 μg), cloxacillin (5 μg), sulphafurazole (100 μg), erythromycin (10 μg), chloramphenicol (10 μg), streptomycin (10 μg), tetracycline(10 μg), gentamycin (10 μg) and kanamycin (30 μg). The plates were then incubated in a hydrogen microaerophilic atmosphere at 37°C and examined after 24 and 48 hr. A zone of inhibition of bacterial growth 10-15 mm around the antibiotic disc after 24 hr incubation was accepted as evidence of sensitivity to that particular antibiotic and the appearance, on the 2nd day, of antibiotic resistant colonies in the clear zone around the disc, was also noted.

Other mucosalis strains listed in Table 1, were also examined in a similar way.

c. Results.

Examination of the CBA plates showed that all the mucosalis strains were sensitive to most of the antibiotics

tested after 24 hr, with the exception of cloxacillin. On the 2nd day, however, a few resistant colonies appeared in the penicillin zone but not with other antibiotics.

d. Comment.

Apart from cloxacillin, which had no effect on mucosalis, and possibly penicillin, the remaining antibiotics might prove useful in eliminating extracellular bacteria in mucosalis infected cell cultures.

B. THE USE OF ANTIBIOTICS TO ELIMINATE EXTRACELLULAR BACTERIA IN C. SPUTORUM SS MUCOSALIS-INFECTED CELL CULTURES.

a. Introduction.

The use of antibiotics in tissue culture media to eliminate extracellular bacteria in infected cell cultures has already been described (Chapter I). Despite considerable controversey over the ability of antibiotics to penetrate living cells and exert an inhibiting effect on the survival of bacteria within the cell, several workers have claimed that intracellular microorganisms are protected from the bactericidal action of non fat-soluble antibiotics, including gentamycin (Lobo and Mandell, 1973; Mandell, 1973). Based on the assumption that gentamycin effects only extracellular bacteria, Kihlstrom (1977) was able to demonstrate an increase in the intracellular bacterial population after gentamycin treatment of HeLa cells infected with the 395 MS and MR 10 strains of S. typhimurium.

In this present study, the results of antibiotic sensitivity tests on a number of strains of C. sputorum ss mucosalis clearly showed that this organism is highly sensitive to many antibiotics including gentamycin.

Other pathogenic bacteria examined in cell cultures do not demonstrate the features of mucosalis infection of cell cultures. C. sputorum ss mucosalis i) lacks the ability to multiply in tissue culture medium in the absence of cultured cells, ii) gives rise to a low level 'parasitic multiplication', not exceeding 10^{3to4} bacteria/ml in permissive cell cultures and, iii) can be incompletely removed by repeated washing of infected monolayers, particularly after the active phase of bacterial attachment (4-6 hr post-inoculation). These findings suggest that mucosalis should be more readily inhibited in the extracellular fluid than other pathogenic bacteria. An attempt was made therefore to eliminate the extracellular bacteria from infected cultures by exposing mucosalis-infected monolayers for short periods of time to different concentrations of gentamycin and to combinations of penicillin and streptomycin.

b. Design of experiment.

i. Preparation of antibiotic solutions.

Stock solution of gentamycin (1000 μg/ml), penicillin (1000 IU/ml) and streptomycin (1000 μg/ml) were prepared in sterile distilled water and filtered through Millipore membrane filters of 0.22 μm A.P.D. They were stored at

4°C until required. Fresh stock solutions of antibiotics were prepared weekly and the final concentrations used are shown under the appropriate heading for each experiment.

ii. Treatment of mucosalis-infected cell cultures.

It will be recalled that the different types of primary and continuous cell cultures used in this study were grown and routinely maintained in antibiotic-free MEM. In this present experiment 24 hr-old confluent monolayers of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK and HeLa cells were grown in 4 oz medical flats. Following inoculation with 10 ml of a suspension of mucosalis strain 253/72 in MEM the cultures were incubated overnight at 37°C. The monolayers were then rinsed in 3 changes of warm PBS to remove unattached extracellular bacteria and reincubated for a further period of 3 hr with fresh MEM containing either gentamycin (10 or 100 µg/ml) or a mixture of penicillin (100 IU/ml) and streptomycin (100 µg/ml). At the end of this period the cell cultures were again thoroughly rinsed in several changes of warm PBS to remove killed bacteria and traces of antibiotics, refed with fresh antibiotic-free MEM containing 5% calf serum and returned to the 37°C incubators. The bactericidal effect of the antibiotics on extracellular mucosalis was checked at 0, 2, 4, 6, 24 and 48 hr by plating 0.2 ml of the supernatant fluids from each cell culture on each of the two CBA plates and incubating them at 37°C in a hydrogen

microaerophilic atmosphere.

As controls, untreated monolayers infected with mucosalis organisms were processed as described above. At this stage no attempt was made to quantify the mucosalis growth in either the control or antibiotic treated cell cultures.

c. Results.

The findings demonstrated that viable mucosalis organisms could be isolated in increasing numbers from the supernatant fluids of all cell lines during the first 48 hr following antibiotic treatment. Similar results were also obtained from all the infected controls.

A subjective assessment of the number of colonies recovered from the supernatant fluids of antibiotic treated cell cultures indicated that fewer mucosalis colonies were obtained from treated cultures compared with those from infected controls. No appreciable difference was found between the inhibiting action of gentamycin and that of the penicillin/streptomycin mixture.

The prolonged exposure (1-10 hr) of mucosalis-infected cell cultures to varying concentrations of gentamycin (10, 20, 100, 500 or 1000 µg/ml) and, to a mixture of penicillin (10, 20, 100, 500 or 1000 IU/ml) and streptomycin (10, 20, 100, 500 or 1000 µg/ml) showed that the antibiotics, irrespective of their concentrations, were unable to eliminate bacteria completely from the supernatant fluids within 5 hr, but did so after 6 hr and onwards.

d. Comment.

The results suggest that the antibiotics used were either unable to produce a bactericidal action or that a number of bacteria were persisting intracellularly. No simple method exists for the distinction between extra- and intracellular bacteria in the infected cell cultures, and mucosalis interaction with the cell cultures appears to be no exception. The assumption that as mucosalis, unlike other pathogenic bacteria, fails to grow in tissue culture medium but produces a low level "parasitic multiplication" in cultured cells and might therefore be readily eliminated from the extracellular fluids by antibiotics proved to be deceptive. Critical confirmation of intracellular multiplication of mucosalis appears to depend in part on successful elimination of extracellular organisms in the infected cell cultures.

It is obvious that the bactericidal effect of antibiotics in this particular experiment appears to depend on the duration of contact rather than their increased concentrations. This is in sharp contrast with the behaviour of other pathogenic bacteria which appear to respond more readily to increased concentrations of antibiotics, thus reducing the actual contact period required to kill the organisms.

Despite the fact that the minimum concentrations of gentamycin (10 μg/ml), or the penicillin (10 μg/ml) and streptomycin (10 μg/ml) mixture, were sufficient to eliminate extracellular mucosalis organisms, the time

taken to bring about this bactericidal effect appears to be unusually long and might result in the penetration and accumulation of these antibiotics within the cell, thus directly influencing the survival of intracellular bacteria.

In view of the practical importance of these factors in the assessment of intracellular multiplication of mucosalis, it was decided to investigate further the minimum time required for these antibiotics to produce a bactericidal action on this organism by the 'replicate plate' method described by Lederberg and Lederberg, (1952).

C. DETERMINATION OF BACTERICIDAL ACTIVITY OF GENTAMYCIN, PENICILLIN AND STREPTOMYCIN ON C. SPUTORUM SS MUCOSALIS ORGANISMS.

b. Design of experiment.

Master plates were prepared by uniformly spreading 0.2 ml of a 24 hr-old mucosalis culture suspended in tryptose phosphate broth (TPB) on the surface of dry CBA plates. To each was then added an 'Oxoid multodisk' containing gentamycin (10 µg), penicillin (2.5 IU) and streptomycin (10 µg), and the plates were incubated at 37°C in a hydrogen microaerophilic atmosphere. At hourly intervals, for 10 hr, one of the 'master plates' with the discs removed, was used to charge a sterile velour pad with the residual inoculum and 'replica plates' were prepared in duplicate on fresh CBA medium. The 'replica plates' were incubated in a hydrogen micro-

aerophilic atmosphere and examined for the presence of surface colonies after 24 and 48 hr. The minimum time required to prevent colony formation on the 'replica plates' was an indication of the relative efficacy of each antibiotic to exert a bactericidal effect.

c. Results.

Examination of the 'replica plates' showed that gentamycin and streptomycin had a bactericidal effect on mucosalis after 5 hr of exposure. With penicillin, however, the inhibitory effect appeared to be bacteriostatic rather than bactericidal since the development of surface colonies was not prevented by this antibiotic during the period of the experiment.

d. Comment.

The fact that 'replicate plate' cultures showed that at least 5 hr of exposure to gentamycin or streptomycin are necessary to bring about a bactericidal effect on mucosalis is in general agreement with the earlier results obtained in cell cultures which also showed that the time taken to inhibit the growth of mucosalis was not affected by the concentration of antibiotics.

The antibiotic 'multodisk' procedure suggested that mucosalis was highly sensitive to a number of antibiotics including gentamycin and streptomycin. Such tests are generally read after 18-24 hr incubation but they do not readily distinguish between bacteriostatic and bactericidal activity. These results, therefore, do not contradict the observation made on the bactericidal activity

of these drugs in tissue culture fluids or by replica plating.

D. EFFECT OF NONIDET-P40 AND SODIUM DEOXYCHOLATE ON THE GROWTH OF C. SPUTORUM SS MUCOSALIS.

a. Introduction.

The disintegration of infected cell cultures to release intracellular bacteria is an important step in the procedures used to quantify intracellular multiplication of pathogenic microorganisms. Several methods have been used by different workers for this purpose, including mechanical disruption in tissue grinders or homogenizers, ultrasonic disruption of infected cells or cell lysis by chemicals such as sodium deoxycholate.

In the present study, attempts were made to find suitable methods of lysing the infected cell cultures in order to release any intracellular bacteria. No attempt was made to disintegrate the infected cells with tissue grinders since it is generally believed that this may result in incomplete disruption of the cells, or by ultrasonic disruption which is difficult to standardise and may produce harmful effects on the viability of C. sputorum ss mucosalis. Instead, sodium deoxycholate (SDC) (Oxoid, London) or a non-ionic detergent, Nonidet-P40 (NP-40) (BDH, London) which lyse cells, were used. As information is not available on the ability of mucosalis to grow in the presence of those substances, a

pilot experiment was carried out to see if either of these substances had any inhibitory effect on this organism.

b. Design of experiment.

i. Preparation of NP-40 and SDC stock solutions.

Stock solutions of NP-40 (5%) and SDC (0.5%) were prepared in PBS and sterilized by autoclaving at 10 pounds/sq. inch for 15 min. Solutions were stored at room temperature until required, and the final concentrations used as given in the appropriate experimental designs.

ii. Growth of mucosalis in the presence of NP-40 and SDC.

Several dry CBA plates were spread uniformly with 0.1 ml of NP-40 (0.1; 0.5; 1.0 or 2 per cent) or SDC (0.05 or 0.5 per cent) and then held at room temperature for 15 min. These plates were then overlaid with 0.1 ml of a suspension of mucosalis strain 253/72 (24 hr-old) in tryptose phosphate broth (approximately 1×10^7 bacteria/ml) and incubated at 37°C in a hydrogen microaerophilic atmosphere. They were examined for the presence of bacterial growth after 24 and 48 hr. Control CBA plates overlaid with 0.1 ml of sterile PBS, followed by 0.1 of mucosalis suspension,were incubated and examined as above.

c. Results.

This experiment attempted to represent the experimental situation whereby potentially viable mucosalis

organisms released from cells would be plated on bacteriological media in the presence of the lytic agent. Subjective assessment of the amount of bacterial growth present on the surface of CBA plates treated with NP-40 and SDC, and on control plates, showed that mucosalis was inhibited by SDC at a final concentration of 0.5% whereas 0.05% had no effect. By contrast, NP-40 appeared to be non-inhibitory at the concentrations used and the amount of bacterial growth was similar to that on the control plates.

Additional tests confirmed that NP-40 was not inhibitory to mucosalis even at a concentration of 5% and subcultures of mucosalis were readily obtained from these plates for upto 10 days after exposure to this detergent. The colonial morphology of mucosalis grown in the presence of NP-40 or SDC was similar to that obtained on untreated CBA plates.

d. Comment.

This experiment clearly shows that treatment of mucosalis grown in culture with NP-40 does not affect the organisms' viability. This property of NP-40 provides a useful means of lysing infected cell cultures without affecting the viability of this organism.

E. PRODUCTION OF CELL LYSIS BY NONIDET P-40.

a. Introduction.

Experimental evidence has suggested that C. sputorum ss mucosalis is not affected by the presence of up to 5% concentration of Nonidet P-40, and the organisms remain viable following this treatment for several days when incubated in an appropriate gaseous atmosphere. At this stage it was considered important to examine the time required by different concentrations of NP-40 to produce lysis of cell cultures, the intention being to induce rapid cell lysis and so avoid unnecessary exposure of extracellular bacteria to possible harmful effects of NP-40.

b. Design of experiment.

After removing the supernatant medium, several 2 to 3-day-old confluent monolayers of BK, BK_{pi}, BHK and HeLa cells grown in 4 oz medical flats were overlaid with 5 ml of a 0.5, 1 or 2% NP-40 solution and incubated at 37°C for 20 to 30 min. The lytic effect of NP-40 on the cell sheet was examined every 5 min using a light microscope.

c. Results.

Lysis of the inoculated cell sheets began soon after exposure to NP-40 and the process appeared to be complete within 5 min in 2%; 5 to 10 min in 1% and 15 to 20 min in 0.5% solutions, with the formation of a tenaceous jelly of lysed cells. This gelatinous material became more compact and formed an insoluble mass when

held at 37°C or at room temperature, and vigorous mixing with a pipette was necessary to disintegrate the gel into fine strands of cell debris. This procedure of breaking-up the gel produced marked 'frothing' of the detergent which took a long time to settle.

In additional experiments it wasfound that the amount of 'frothing' could be greatly reduced by using the smallest concentration of NP-40 (0.1%) necessary to lyse the cells, followed by gentle and steady mixing as soon as disintegration of the cell sheet had occurred.

IV. A. ISOLATION OF INTRACELLULAR C. SPUTORUM SS MUCOSALIS AFTER ELIMINATION OF EXTRACELLULAR 'PARASITIC GROWTH' WITH ANTIBIOTIC TREATMENT.

a. Introduction.

The method of assessing intracellular multiplication of pathogenic bacteria in infected cell cultures is basically a three step procedure, viz:

i. elimination of extracellular bacteria by the use of appropriate antibiotics,

ii. disintegration of infected cells to release intracellular bacteria and

iii. isolation of these organisms on suitable bacteriological media.

It will be recalled that in the present series of experiments suitable antibiotic techniques were developed to eliminate extracellular bacteria, and NP-40 treatment

was used to disintegrate infected cell cultures for the release of intracellular bacteria. The isolation of intracellular bacteria from lysed cell homogenates is a routine bacteriological procedure so far as C. sputorum ss mucosalis is concerned. For these reasons the following experiment was devised to assay the intracellular bacteria in cell cultures previously treated with antibiotics to remove any extracellular bacteria.

b. Design of experiment.

In this experiment 8 different types of cell cultures (PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK, DK and HeLa) were grown overnight in 4 oz flats. Each was overlaid with 10 ml of a suspension of mucosalis (strain 253/72) in MEM (approximately 1×10^7 bacteria/ml). After overnight incubation at 37°C, the infected monolayers were rinsed in 3 changes of warm PBS to remove unattached extracellular bacteria and reincubated for another 5 hr in MEM containing gentamycin (10 µg/ml). In addition, a number of parallel experiments were carried out using streptomycin (10 µg/ml) to eliminate extracellular bacteria. At the end of the 5 hr period the monolayers were thoroughly rinsed in several changes of PBS to remove dead bacteria and traces of antibiotics, and were returned to the incubator after refeeding with 10 ml of antibiotic free MEM. Two medical flats of each infected cell line previously treated with gentamycin or streptomycin were removed daily for 10 days and the monolayers 'lysed' separately by adding 1 ml of 1% NP-40 to the

supernatant medium. These were then held for 15 min at 37°C and each of the cell lysates was individually homogenized by gentle mixing with a sterile pipette. Several dry CBA plates were spread uniformly with 0.2 ml of each of the cell lysates and the inoculated plates were then held at room temperature prior to incubation at 37°C in a hydrogen microaerophilic atmosphere. The plates were examined daily for evidence of mucosalis growth and were considered to be negative if no bacterial growth occurred within 3 days of incubation.

Several cultures of each cell type infected with mucosalis, but untreated with antibiotics, were included as controls and were processed and examined as above.

c. Results.

Examination of CBA plates inoculated with the homogenates of 8 different types of infected cell lines obtained after antibiotic treatment with either gentamycin or streptomycin, failed to show the growth of mucosalis organisms. By contrast, mucosalis organisms were regularly recovered from the control cultures of pig kidney cell lines for up to 4-5 days and from controls of all other cell types throughout the whole period of the experiment (10 days).

d. Comment.

The inability to recover mucosalis from infected cells following a short period of exposure to gentamycin or streptomycin is in marked contrast to the situation in untreated cell cultures and clearly shows that the

assessment of intracellular growth of this organism is by no means a simple procedure. Several questions remain unanswered; it is not clear,for example,whether inactivation of intracellular organisms is associated with some unknown host cell defence mechanism or if it is due to penetration and accumulation of significant levels of antibiotics within the cells.

It is generally believed that many pathogenic microorganisms can be inactivated within the host cell and in this respect mucosalis may be no exception.

In the present experiment, the time required (5 hr) by gentamycin and streptomycin to produce the bactericidal action on mucosalis appears to be unusually long and may provide sufficient time for the antibiotic to be absorbed into the host cell. This would result in death of the intracellular bacteria and account for the failure to recover viable mucosalis from infected cell cultures following treatment with antibiotics. In 1977, Kihlstrom reported that the absorption of gentamycin into HeLa cells resulted in a reduction in the recovery rate of intracellular S. typhimurium and that the effect appeared to be time and dose dependent. In this present investigation however, the fact that the bactericidal action of gentamycin and streptomycin on mucosalis is time rather than dose dependent, greatly restricts the scope of experimental procedures designed to reduce the absorption of antibiotics into the cell. However, it may be possible to overcome these difficulties by using

shorter-acting antibiotics which can eliminate extracellular organisms before being absorbed by the cell. Until such investigations are carried out it cannot be claimed that mucosalis is unable to survive and multiply within the cell, nor would it be wise to do so until attempts have been made to demonstrate the intracellular multiplication of mucosalis in the infected cell cultures by other techniques.

B. QUANTITATIVE ASSESSMENT OF 'TOTAL PARASITIC GROWTH' OF C. SPUTORUM SS MUCOSALIS IN CELL CULTURES.

a. Introduction.

Since treatment of infected cell cultures with gentamycin or streptomycin eliminated viable C. sputorum ss mucosalis, the method is not suitable for evaluating possible intracellular multiplication of this organism. Further developments along these lines must await confirmation of intracellular multiplication by other methods including the use of antibiotics capable of destroying only extracellular mucosalis.

Until such procedures are available, the assessment of growth and development of mucosalis within susceptible host cells is likely to be largely dependent on light, ultraviolet and electron microscopic studies of cell cultures at different stages of infection; although such studies may not by themselves be entirely conclusive.

In view of the rapid death of mucosalis in cell-free media and in non-permissive cell types, the isolation of mucosalis organisms from 'permissive' cells over a period of time could indicate intracellular bacterial persistence. A more detailed study of this observation might show whether mucosalis actually multiplies intracellularly or if its recovery from these cell cultures is merely due to the persistence of a declining bacterial population without multiplication.

Because it has been difficult to differentiate intracellular from cell-associated extracellular growth of mucosalis, the following experiment was designed to determine the 'total yield' of viable mucosalis organisms irrespective of whether they have multiplied within or outwith the infected cells. Such information would be of value in interpreting the quantitative data provided by the viable counts of the supernatant cell culture fluids carried out in Expt. II, p.114, (Table 6).

b. Design of experiment.

It should be emphasized at this stage that the essential difference between this experiment and the quantitative assay experiment described previously is that in the latter case recovery of bacteria was assessed from the supernatant fluids whereas in the present experiment an assessment will be made of the 'total yield' of both intra- and extracellular bacteria.

i. Inoculation of cell cultures.

Confluent monolayers of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, HeLa and BHK cells grown overnight in 4 oz medical flats were overlaid with 10 ml of a suspension of mucosalis strain 253/72 in prewarmed (37°C) MEM and incubated at 37°C. The number of organisms present in the inoculum was determined by the surface viable count technique.

Two infected monolayers of each cell type were examined daily during the first 5 days and on the 7th, 14th and 21st day post-inoculation, after lysing the cells by the addition of 1 ml of 1% NP-40 directly to the supernatant tissue culture fluids. The NP-40 treated medical flats were incubated at 37°C for 15 to 20 min and the lysates were then gently homogenized with a 10 ml pipette. The cell homogenates were used to assay 'total viable' mucosalis organisms by the surface viable counts technique (vide supra).

As controls, several CBA slopes were overlaid with 10 ml of a suspension of mucosalis strain 253/72 in prewarmed (37°C) MEM, and the rate of bacterial growth in this diphasic medium was compared with that obtained following lysis of the infected cell cultures. The inoculated CBA-MEM diphasic slopes were then divided into two groups of four, and one set was incubated at 37°C under 'aerobic' conditions whilst the other was incubated in a hydrogen microaerophilic atmosphere. Viable counts from the diphasic cultures were made daily for 5 days after inoculation, as described above.

Additional experiments were carried out to assess the persistent growth of mucosalis in BK, BK_{pi} and OK_{pi} cells over a period of 12 weeks. These cell types were selected as they appeared to be less permissive than the others, and did not show the marked destructive cellular changes observed in PK and PK_{pi} cells. These three cell lines were refed at weekly intervals and subcultured every three weeks by trypsinization with STV. Viable counts of mucosalis organisms were obtained at weekly intervals after lysis of cell sheets with NP-40, by the method described above.

c. Results.

The results of the viable counts (Table 7) indicate that there was little difference in the 'total yield' of viable bacteria from different cell types during the first 5 days, and that the counts compared favourably with those of bacteria grown in CBA-MEM diphasic medium in a hydrogen microaerophilic atmosphere. There was no evidence of bacterial growth in CBA-MEM diphasic medium incubated under 'aerobic' conditions. The persistence of mucosalis in BK, BK_{pi} and OK_{pi} cells for upto 5 to 7 weeks confirmed earlier observations on 'parasitic growth' of this organism.

d. Comment.

Considering the fact that mucosalis is rapidly killed within 12 hr under 'aerobic' growth conditions in cell-free MEM, the results of this experiment clearly indicate that active multiplication of bacteria has occurred in the

TABLE 7

ISOLATION OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72

FROM CELL CULTURES AFTER LYSIS WITH NONIDET P-40.

Hours (h), days (d) or weeks (w) after inoculation.	Cell types examined					Controls	
						CBA+MEM	Cell-free MEM
	PK	PK_{pi}	BK	BK_{pi}	OK_{pi}	H_2+CO_2 incubation	Aerobic incubation
0h	6.70*	6.70	6.70	6.70	6.70	6.70	6.70
1d	6.78	5.70	4.70	6.78	4.70	8.15	-
2d	6.81	6.31	6.60	6.48	6.30	8.45	-
3d	6.18	6.35	6.78	6.48	6.40	9.70	-
4d	6.02	6.48	6.18	6.18	6.18	8.36	-
5d	5.18	6.48	6.18	6.55	6.70	7.38	NT
1w	-	-	5.70	4.70	4.18	NT	NT
2w	-	-	5.30	4.30	2.48	NT	NT
3w	-	-	4.60	3.00	2.10	NT	NT
4w	-	-	4.39	2.10	a	NT	NT
5w	-	-	3.39	a	a	NT	NT
6w	-	-	a	a	a	NT	NT
7w	-	-	a	-	-	NT	NT
8w	-	-	-	-	-	NT	NT

* = Bacterial counts expressed as organisms $\log_{10}$/ml, corrected to the second decimal place.

a = A few colonies of mucosalis invariably present in undiluted cell lysates.

\- = No bacterial growth.

NT = Not tested.

infected cell cultures. Of particular interest is the fact that the "total yield" of bacteria obtained after lysis of infected cells is undoubtedly much higher than that from supernatant fluids only. Since this clearly indicates that mucosalis organisms isolated after lysis of cells are produced by intracellular multiplication, further investigations by way of light, ultraviolet and electron microscopy should be carried out to confirm this important finding.

DISCUSSION

The 'parasitic growth' of C. sputorum ss mucosalis in cultured cells is an unusual and interesting phenomenon, and it is therefore important to establish whether the bacteria involved are merely persisting in the cells or are increasing in numbers and, if so, at which site multiplication is taking place.

So far as 'parasitic growth' in cell cultures is concerned, there appears to be no parallel situation with other species of pathogenic bacteria. Unlike mucosalis, most other bacteria that have been examined in cell cultures are those that grow freely in cell-free media or fail to grow in the absence of tissue cells (e.g. rickettsiae).

Since very little information is available about the 'parasitic growth' of other pathogenic bacteria in cell cultures, it is difficult to make deductions from the findings of other workers which might explain the unusual

growth characteristics of mucosalis. However, the salient features of 'parasitic growth' of mucosalis can be summarised as follows:

i. A quantitative assay of mucosalis in supernatant culture fluids has indicated that a marked initial drop in the number of extracellular bacteria takes place during the first 24 hr which is followed by a gradual increase. These changes are associated with persistence of bacteria in the supernatant fluids for prolonged periods even when the medium is unchanged or the cultures are serially passaged. However, these procedures undoubtedly produce a decrease in the number of bacteria in the supernatant fluids.

The most important fact that emerges from this experiment is that 'parasitic' bacteria appear to be released from infected cells following a stage of intracellular multiplication and that it is unlikely that prolonged persistence of 'parasitic growth' is the result of extracellular multiplication.

ii. Elimination of extracellular bacteria by gentamycin or streptomycin treatment of infected monolayers is difficult to achieve since it leads to the death of both intra- and extracellular mucosalis. Therefore attempts to assess the growth and development of intracellular bacteria alone have been unsuccessful

and alternative antibiotic procedures need to be developed if selective elimination of extracellular organisms is to be achieved.

iii. Because it has not been possible to quantify the intra- and extracellular bacteria separately, an attempt was made to obtain a "total count" of the viable bacteria, irrespective of their location within or outwith the cell, after lysis of the infected monolayers. This technique was of value and the results provided further evidence in support of intracellular growth.

iv. A comparison of the viable counts of mucosalis showed a marked increase in the yields of bacteria following lysis of infected cells compared with those in supernatant fluids. This undoubtedly suggests that mucosalis is able to survive and multiply intracellularly over prolonged periods. Also at this stage it has to be presumed that intracellular growth might be the source of extracellular mucosalis which is released into the supernatant fluids following destruction of the infected cells or by other means which are not understood.

In conclusion, the present findings suggest that 'parasitic growth' of mucosalis occurs over a prolonged period in certain types of cell cultures and appears to be due primarily to intracellular multiplication and subsequent release of these organisms into the supernatant cell culture fluids, possibly from dead or dying infected cells.

CHAPTER VI

CHAPTER VI

ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO CELL CULTURES.

GENERAL INTRODUCTION.

Colonization of host tissues is the first step in the development of a disease process and, with pathogenic bacteria, this often occurs first on the mucosal surfaces such as those of the respiratory, gastrointestinal and urinary tracts. For colonization to take place, the bacteria must first attach firmly to the mucosal surfaces. Later, they may invade the host tissues or synthesize their toxins and thus cause disease. In the absence of attachment the organism may be eliminated by a variety of host defence mechanisms and be unable to induce disease.

The first clear evidence of the importance of bacterial adhesion was reported in 1972 when Jones and Rutter showed that certain porcine strains of E. coli produced not only toxins but also an antigen, designated K88, which enabled these organisms to attach to the surfaces of intestinal cells and thereby cause diarrhoea in piglets. It is now generally believed that other pathogenic bacteria possess similar mechanisms for their adhesion to host cells (e.g. K99, pili, etc.) and that attachment is the first step in the infectious process. The subject has been extensively reviewed by several workers notably Gibbons and van Houte, 1975; Jones, 1977; Smith, 1977; Arbuthnott and Smyth, 1979 and Gibbons, 1980.

In recent years, the phenomenon of bacterial attachment to cell surfaces has attracted the attention of increasing numbers of investigators mainly because its specificity provides a basis for understanding the innate resistance of the host and, in particular, of their tissues to bacterial infection. However, investigations of the ability of pathogenic bacteria to attach to host tissues in vivo are not without inherent drawbacks. These include spread of artificially-induced infection, the presence of immune and other non-specific responses of the host and inability to induce readily the disease state in experimental hosts. In view of these practical difficulties several investigators have turned to cell cultures as an alternative means of studying the mechanisms involved in host-parasite interactions and a brief description of the pathogenic bacteria that have been studied by these means is given in Chapter I.

Although C. sputorum ss mucosalis has been regularly demonstrated in the cytoplasm of adenomatous intestinal epithelium (Rowland and Lawson, 1974; Roberts, 1978), very little information is available about the mechanisms responsible for infection, including the adhesive properties of the organism. For this reason, the following series of experiments were undertaken to examine the adhesive properties of mucosalis organisms in different types of primary and established cell cultures. Such studies were seen as an essential step towards understanding some of the mechanisms involved in successful

intracellular multiplication of this parasite.

A. ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO YEASTS AND RED BLOOD CELLS.

a. Introduction.

This preliminary study was undertaken to examine the adhesive properties, if any, of C. sputorum ss mucosalis to red blood cells and yeasts, as a model for subsequent studies on host-cell relationships with mucosalis.

b. Design of experiment.

The procedures for obtaining suspensions of red blood cells (RBCs) of pigs, cattle and horses have been described in Chapter II. An homogeneous thin suspension of a laboratory strain of C. albicans was prepared in sterile PBS from a 3 to 4-day-old surface growth on CBA. In each case, the suspensions were prewarmed to 37°C before use. The bacterial inocula consisted of 24 hr-old diphasic growths of mucosalis (strains 253/72 and 982/76) diluted (1:5) in warm (37°C) tryptose phosphate broth (TPB). Equal volumes (1 ml) of suspensions of each species of RBCs were separately mixed with a strain of mucosalis and incubated in test tubes at 37°C. At hourly intervals during the first 5 hr of incubation, 0.2 ml of the cell suspensions was withdrawn and the cells spun at 1000 x g for 5 min on to a clean glass slide in a cyto-centrifuge (Shandon Co., England). The cell smears were then fixed in acetone, stained by Giemsa's method and examined. Yeast cell suspensions were examined similarly. In selected experiments, the cell suspensions were also examined by phase contrast microscopy.

c. Results.

Giemsa stained cyto-centrifuge preparations of pig, cattle and horse RBCs exposed to mucosalis failed to show bacterial attachment to the cell surfaces, and similar findings were obtained with yeast cells. These preliminary observations clearly indicated that mucosalis is unable to attach to either red blood or yeast cells in such an experimental system. By phase contrast microscopy it was also possible to demonstrate actively motile bacteria striking the cell surfaces of RBCs and yeast cells although they failed to attach firmly to these cells. Non-motile bacteria were often seen in close proximity to RBCs or yeast cells, and no evidence of the attachment of such organisms was observed.

Additional experiments were conducted by incubating the inoculated cell suspensions in air at room temperature ($22^{\circ}C$) and in the presence of 5% CO_2 at 22° and $37^{\circ}C$. In each case stained cyto-centrifuge preparations failed to show bacterial attachment and, interestingly, phase contrast microscopy showed greatly reduced bacterial motility in suspensions incubated at $22^{\circ}C$ in air as well as 5% CO_2.

d. Comment.

Although mucosalis failed to attach to yeasts or different species of RBCs, the experiment was successful in that the results pin-pointed the possible importance of active motility in enhancing bacterial adhesion and that this activity might be related to the temperature of incubation. Therefore, care was taken in all subsequent

experiments to bring the cell cultures, media and other reagents to 37°C prior to use.

B. ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO THE SURFACES OF CULTURED CELLS.

a. Introduction.

Several workers have shown that a variety of pathogenic bacteria are able to attach to cell cultures, and the pertinent literature has been reviewed in Chapter I.

In the present study, preliminary attempts were made to demonstrate attachment of C. sputorum ss mucosalis to preformed monolayers of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cell lines. Of particular interest was the use of PK and PK_{pi} cells since these might be expected to have receptor sites similar to those of naturally occurring pig epithelial cells. The PK_{pi}, BK_{pi} and OK_{pi} cells were included not only because the three different species of cells were readily available in the laboratory but because the great majority (>95%) of the cells have altered surface cell membranes due to the presence of a persistent infection with a membrane-forming virus namely Newcastle disease virus (NDV) (Fraser et al., 1976). It was thought that the modified plasmalemma of these chronically infected epithelial cells might favour attachment and penetration of bacterial cells and provide useful information on the mechanisms involved in mucosalis-host cell relationships. The following experiment was designed to investigate some of the adhesive properties of different strains of mucosalis, E. coli and C. coli on these various cell cultures.

b. Design of experiment.

i. Preparation of bacterial inocula.

The species of bacteria used in this experiment were; mucosalis strains 253/72; 982/76; 512/77; 1075/78; enteropathogenic E. coli strains O149:K91,K88ac; O141: K85,K88ac and C. coli, 124/73 A4. Also included was a biochemically and serologically distinct, catalase-negative Campylobacter strain 20/74. Procedures for the routine growth and maintenance of these organisms on bacteriological media have been described previously.

Bacterial suspensions were prepared from 24 hr-old CBA slope cultures of each of these organisms by flooding them with 10 ml of prewarmed (37°C) MEM according to the method described in Chapter IV (p. 95).

ii. Inoculation of cell cultures.

Preformed (24 hr-old) coverslip cultures of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cells, obtained by routine cell culture procedures were overlaid with 1 ml of a suspension of each bacterial strain and incubated at 37°C. These, and appropriate uninfected control cultures, were examined at hourly intervals during the first 10 to 12 hr and at 24 and 48 hr post-inoculation. After thorough rinsing in several changes of PBS to remove unattached bacteria, the monolayers were fixed in methanol and stained with Giemsa. In selected experiments coverslips were fixed in acetone and stained by Gimenez' (1961) method.

iii. A semi-quantitative assessment of bacterial attachment.

In selected experiments, a semi-quantitative assessment of the attachment of mucosalis strain 253/72 was

attempted in Giemsa-stained coverslip monolayers obtained at 2, 4, 6, 8, 10, 12, 24 and 48 hr post-inoculation. At least 100 cells were examined at random in each coverslip for the presence of attached bacteria, and the numbers on each cell were noted. Since bacterial attachment is a dynamic process, all cells may not demonstrate a uniform number of attached bacteria at a particular time. In view of this, the cells were arranged into 4 groups on the basis of bacterial attachment namely of 1 to 10, 11 to 20, more than 20 organisms or no attachment per cell. The largest group of cells showing a particular pattern of bacterial attachment at a given time was taken as a representative picture of the adhesive process.

c. Results.

i. Evaluation of staining methods for detecting mucosalis in cell cultures.

Staining of coverslip cultures by Gimenez' method imparted a bright red colour to mucosalis organisms against a bluish green background of the cytoplasm and was preferred to Giemsa's method for the recognition and differentiation of vibrioid bacteria from the reticulated cytoplasm and other constituent processes of the host cell. However the results obtained with Gimenez' stain were often inconsistent and its usefulness depended on critical 'destaining' of carbol fuchsin with fast green, which could only be determined by direct microscopical examination of wet preparations. Well stained preparations showed a detail and clarity not obtained

by Giemsa's stain and provided useful confirmatory evidence regarding the relationships between mucosalis and host cells. By comparison, routine Giemsa staining was less tedious but recognition of mucosalis organisms was more difficult since the bacteria did not contrast well against the host-cell cytoplasm.

ii. Demonstration of the attachment of mucosalis to cell cultures.

Giemsa stained preparations of PK, PK_{pi}, BK, BK_{pi} and OK_{pi} cells obtained after 1 hr exposure to each of the mucosalis strains 253/72, 982/76, 512/77 and 1075/78, showed bacterial attachment to the cell surfaces of about 25% of the cell population. At this stage relatively few organisms (less than 10 per cell) were attached to the cell surfaces. Assessment over a period of time indicated that mucosalis attachment reached a maximum around 4 to 6 hr post-inoculation and was maintained at this level for 2-3 hr. During this 'active' phase of attachment, large numbers of bacteria were present on the cell surfaces, and the effect was particularly marked at the periphery of the cells. Almost all the cells were affected and some of those in the process of division showed greater attachment than others. However, this phase was soon followed by a sharp decrease in the number of adhering bacteria until by the end of 10 to 12 hr, only occasional organisms were seen in less than 20% of the cell population. Mucosalis organisms could not be demonstrated on cell surfaces after 24 hr,

TABLE 8

A SEMI-QUANTITATIVE ASSESSMENT OF THE ATTACHMENT OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72 TO CELL CULTURES.

Incubation period in hours	Cell types examined				
	PK	PK_{pi}	BK	BK_{pi}	OK_{pi}
2	+	++	+	++	++
	(50)	(58)	(25)	(50)	(52)
4	++	+++	+	++	++
	(82)	(100)	(45)	(76)	(89)
6	+++	+++	++	+++	+++
	(100)	(100)	(66)	(85)	(90)
8	+++	+++	++	+++	+++
	(100)	(100)	(70)	(90)	(90)
10	++	++	+	++	++
	(50)	(60)	(30)	(35)	(40)
12	+	+	+	+	+
	(10)	(20)	(5)	(10)	(20)
24	-	-	-	-	-
	(0)	(0)	(0)	(0)	(0)
48	-	-	-	-	-
	(0)	(0)	(0)	(0)	(0)

\+ = Attachment predominently with less than 10 bacteria/cell.

++ = Attachment predominently with 11 to 20 bacteria/cell.

+++ = Attachment predominently with 21 or more bacteria/cell.

\- = No bacterial attachment.

Figures in parenthesis indicate the percentage of cells showing attached bacteria (>1 bacteria/cell).

although most cells contained what appeared to be 'poorly stained' intracellular bacteria (Table 8).

A comparative evaluation of bacterial attachment to different types of cell cultures observed during the peak period (4 to 6 hr) indicated that greater numbers of bacteria attached to PK_{pi} than to other types of cell lines, but that PK, BK_{pi} and OK_{pi} cells were more active than BK cells in this respect (Table 8).

iii. Attachment of other bacteria.

Unlike mucosalis strains, other bacterial species including E. coli, C. coli and Campylobacter strain 20/74 failed to attach to the surfaces of PK, PK_{pi}, BK, BK_{pi}, and OK_{pi} cells.

d. Comment.

These observations clearly indicate that different strains of mucosalis are capable of attachment to kidney cell lines derived from pig, cattle and sheep, and that the adhesive process is specific since extracellular bacteria are rarely observed in the intercellular spaces.

Although the precise mechanisms involved in the attachment of mucosalis to cell cultures are not understood, the adhesive properties of this organism appear to be unusual in that it is 'transitory' and lasts for about 10 to 12 hr post-inoculation. Although earlier evidence (Chapter IV and V) has shown that mucosalis is invariably present extracellularly for varying periods, it is interesting to note that bacterial attachment cannot be demonstrated at 24 hr and thereafter. Such restricted attachment of mucosalis cannot be explained

at this stage and should be investigated further. The fact that mucosalis attached in greater numbers to cell cultures that are persistently infected with Newcastle disease virus (NDV) suggests that host-cell membranes may play an important role in bacterial attachment and also merits further investigation.

C. ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO OTHER CELL TYPES.

a. Introduction.

The experimental evidence has shown that a variety of cell cultures e.g. PK, PK_{pi}, BK, BK_{pi} and OK_{pi} not only supported 'parasitic growth' of C. sputorum ss mucosalis strains but also possessed specific cell surface receptors for attachment of this organism. For these reasons it was considered important to investigate the adhesive properties of mucosalis in a wide range of cell cultures obtained from different species of animals.

b. Design of experiment.

i. Attachment of mucosalis to preformed monolayers.

Overnight coverslip preparations of PK, PK_{pi}, BK, BK_{pi}, OK_{pi} BK, BHK and HeLa, Vero and $LLCMK_2$ cell lines and 3 to 4 day-old primary cultures of pig kidney (PPK) and chicken embryo fibroblasts (CEF) were inoculated with one of the following strains of mucosalis: 253/72, 982/76, 512/77 or 1075/78. The experimental procedures were similar to those described in the previous experiment and a number of uninfected cultures were included as controls.

ii. Attachment of mucosalis to trypsinized cells.

Seven-day-old monolayer cultures of the above cell types were trypsinized with STV and the detached cells (10^6 cells/ml) were then mixed with 1 ml of a suspension of the appropriate strains of mucosalis (vide supra). The inoculated trypsinized cells were allowed to form monolayers on coverslips at 37°C and appropriate uninfected cultures were used as controls.

iii. Examination of coverslip preparations.

After thoroughly rinsing the cultures in several changes of warm PBS to remove unattached bacteria, the coverslip monolayers were fixed in methanol and stained with Giemsa. Uninfected controls cultures were similarly examined.

c. Results.

It can be seen from the results in Tables 8 and 9 that strain 253/72, as with all other mucosalis strains, were able to attach to the surfaces of PPK, PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, DK, BHK and HeLa cells whether or not they were inoculated before or after the monolayers had formed. The process of bacterial attachment appeared to be 'transitory' and followed a uniform pattern common to all of these cell types. When mucosalis attachment reached its maximum around 4 to 6 hr post-inoculation, it remained at this level for the next 2 or 3 hr, thereafter a rapid decrease in the number of adhering organisms occurred during the next 10-12 hr. There was no evidence of bacterial attachment by 24 hr post-inoculation nor at any time thereafter during the 7 days of this experiment.

TABLE 9

A SEMI-QUANTITATIVE ASSESSMENT OF THE ATTACHMENT OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72 TO CELL CULTURES.

Incubation period in hours	Cell types examined						
	PPK	CEF	DK	BHK	HeLa	Vero	$LLCMK_2$
2	++ (50)	- (0)	+ (10)	++ (50)	++ (55)	- (0)	- (0)
4	++ (72)	- (0)	+ (30)	+++ (80)	+++ (82)	- (0)	- (0)
6	+++ (90)	- (0)	++ (52)	+++ (85)	+++ (85)	- (0)	- (0)
8	+++ (90)	- (0)	++ (50)	++ (46)	++ (37)	- (0)	- (0)
10	++ (45)	- (0)	+ (30)	+ (30)	++ (31)	- (0)	- (0)
12	+ (15)	- (0)	+ (5)	+ (10)	+ (17)	- (0)	- (0)
24	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)
48	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)

\+ = Attachment predominently with less than 10 bacteria/cell.

++ = Attachment predominently with 11 to 20 bacteria/cell.

+++ = Attachment predominently with 21 or more bacteria/cell.

\- = No bacterial attachment.

Figures in parenthesis indicate the percentage of cells showing attached bacteria (>1 bacteria/cell).

Lines of Vero and $LLCMK_2$ cells and primary cultures of CEF failed to show bacterial attachment either in preformed monolayers or in suspensions of trypsinized cells. These unusual findings were confirmed on several occasions and a second culture of Vero cells obtained by courtesy of the Moredun Institute, Edinburgh behaved similarly.

Subjective assessment of bacterial attachment in preformed monolayers during the peak period confirmed an earlier observation that mucosalis attachment was greater in PK_{pi} than in PK or PPK cells, whereas OK_{pi} and BK_{pi} were invariably better than BK, BHK and HeLa cells in this respect. By comparison, DK cells showed limited adhesive properties for this organism.

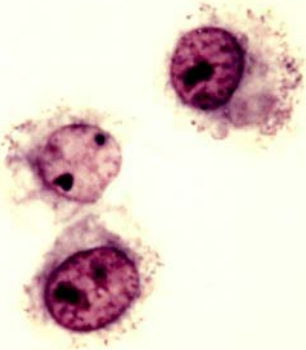
Examination of stained coverslip cultures obtained from trypsinized PPK, PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK and HeLa cell suspensions inoculated with mucosalis indicated that this modified cell infection procedure produced greater bacterial attachment during the first 4 to 6 hr than was achieved by conventional inoculation of preformed monolayers (Fig. 8). However, despite this improvement, the bacteria remained attached to the cell surfaces for only 10 to 12 hr and could not be demonstrated after 24 hr post-inoculation.

d. Comment.

The findings of this experiment clearly showed that C. sputorum ss mucosalis is capable of attachment to a wide range of cells including primary cultures of pig kidney cells (PPK), but not to Vero and $LLCMK_2$ cell

Fig. 8

Six-hour-old coverslip preparation of PK_{pi} cells inoculated with C. sputorum ss mucosalis at the time of seeding. Notice extensive bacterial attachment to the periphery of the cells. There is no evidence of intracellular bacteria. Giemsa X 1500.



lines nor to primary cultures of chicken embryo fibroblasts (CEF). It is also of interest that the duration of attachment was similar in all types of 'permissive cell cultures' irrespective of their species of origin and reached its maximum around 4 to 6 hr but could not be demonstrated after 10 to 12 hr post-inoculation.

The mechanisms involved in bacterial adhesion are not fully understood and warrant further detailed investigation. Failure of mucosalis to attach to Vero, $LLCMK_2$ and CEF cells implies that they differ from other types of cells and lack the receptor sites necessary for mucosalis attachment. The reasons for the disappearance of attached bacteria from the surfaces of 'permissive cells' may prove complex and could indicate either a change in the host cell membrane or some alteration in the bacteria remaining in the supernatant fluids which reduces, destroys or otherwise inhibits their adhesive properties.

The fact that bacterial attachment was greater in trypsinized cell cultures than in corresponding preformed monolayers suggests that the host cell receptors are not destroyed by trypsin. The reason for this improved attachment is not known but it could merely be due to an increase in the ratio of mucosalis organisms per suspended cell. Such cells have not yet undergone any multiplication and are therefore in smaller numbers, a situation not provided in preformed monolayers. Apart from this, the technique of inoculation of suspensions of trypsinized cells seems to hold promise for further attempts to develop cell-infection procedures with this organism.

D. EFFECT OF RE-INFECTION ON THE ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO CEF, VERO AND LLCMK$_2$ CELLS.

a. Introduction.

Earlier observations showed that re-infection of Vero and LLCMK$_2$ cell lines, but not CEF cells, with C. sputorum ss mucosalis produced 'parasitic growth'. Since this was achieved in the two cell lines previously thought to be refractory to the infection, the following experiment was carried out to see whether this form of induced 'parasitic growth' is accompanied by, or in any way associated with, bacterial attachment to cell surfaces.

b. Design of experiment.

The methods employed for primary infection of preformed monolayers and trypsinized cell suspension of Vero, LLCMK$_2$ and CEF cells with mucosalis strains 253/72, 982/76, 512/77 and 1075/78 have already been described (Chapter IV).

After 3 days' incubation at 37°C, the mucosalis-infected coverslips were rinsed in two changes of PBS to remove unattached bacteria, superinfected with 1 ml of the same strain of mucosalis and returned to the incubator. Coverslips were removed at hourly intervals during the first 12 hr, and then at 24 and 48 hr, and examined by the method described on page 150. Appropriate once-infected coverslips were included as controls and were similarly examined.

TABLE 10

A SEMI-QUANTITATIVE ASSESSMENT OF THE ATTACHMENT OF C. SPUTORUM SS MUCOSALIS STRAIN 253/72 TO 'RE-INFECTED' CEF, VERO AND LLCMK$_2$ CELLS.

Incubation period in hours	Cell types examined					
	CEF		Vero		LLCMK$_2$	
	Once-infected	Re-infected	Once-infected	Re-infected	Once-infected	Re-infected
2	- (0) *	- (0)	- (0)	++ (15)	- (0)	++ (13)
4	- (0)	- (0)	- (0)	++ (40)	- (0)	++ (39)
6	- (0)	- (0)	- (0)	++ (50)	- (0)	++ (55)
8	- (0)	- (0)	- (0)	++ (24)	- (0)	++ (23)
10	- (0)	- (0)	- (0)	+ (5)	- (0)	+ (8)
12	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)
24	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)
48	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)

* = Figures in parenthesis indicate the percentage of cells showing attached bacteria (> 1 bacteria/cell).

++ = Attachment predominently with 5 to 10 bacteria/cell.

+ = Attachment predominently with 1 to 4 bacteria/cell.

- = No bacterial attachment.

c. Results.

The data shown in Table 10 indicate that re-infection of Vero and $LLCMK_2$ cells produces limited bacterial attachment. However, not more than 5 to 10 organisms were present on each cell and almost 50% of the cell population remained free of demonstrable organisms during the first 4 to 6 hr. On further incubation there was a rapid fall in the number of adhering organisms until by the end of 10 hr post-inoculation almost all the cells appeared to be free of organisms. There was no significant difference in the degree of bacterial attachment on preformed monolayers and trypsinized cells infected with different strain of mucosalis. 'Once-infected' control cultures of Vero and $LLCMK_2$ cells did not show bacterial attachment during the course of the experiment. At no stage did CEF cells permit bacterial attachment following primary infection or after superinfection with mucosalis.

d. Comment.

The above findings indicate that reinfection of Vero and $LLCMK_2$ cells with mucosalis results in limited bacterial attachment and thereby substantiate the earlier suggestion that 'parasitic growth' of mucosalis may occur following re-infection (Chapter IV).

E. EFFECTS OF INCUBATION TEMPERATURES ON THE ADHESIVE PROPERTIES OF C. SPUTORUM SS MUCOSALIS.

a. Introduction.

C. sputorum ss mucosalis is an actively motile organism (Lawson and Rowland, 1974) and its motility appears to be

greatly influenced by the temperature of incubation. Phase contrast microscopy of liquid cultures shows that mucosalis strains are actively motile at 37°C but less so after exposure to air and lower temperatures.

The importance of motility in enhancing the adhesive properties of several pathogenic bacteria, including Vibrio species, has been well documented (Chapter I) but such information is not available for mucosalis. Because non-motile variants of this organism have not been reported (Dr Lawson, personal communication) it was decided to examine the adhesive properties of mucosalis in infected cell cultures at different temperatures.

b. Design of experiment.

Mucosalis infection of one-day-old preformed monolayers of PK, PK_{pi}, BK and BK_{pi} cells was carried out by the methods described earlier (p.150). The bacterial suspension (strain 253/72) and the coverslip cultures were pre-incubated at 22°, 37°, or 41°C for 30 min prior to replacing the supernatant medium with the mucosalis inoculum. Several inoculated and uninfected control cultures were returned to appropriate incubation temperatures (22°, 37° or 41°C) and Giemsa stained preparations were examined at hourly intervals during the first 8 hr post-inoculation.

c. Results.

Monolayers incubated at 37°C showed marked attachment of mucosalis to PK, PK_{pi}, BK, BK_{pi} cells and the overall picture was similar to that described on page 156. By

comparison, monolayers incubated at 22°C showed poor attachment which appeared to be restricted to about 10% of the cell population, while those cultures incubated at 41°C were completely negative. Phase contrast microscopy of the supernatant fluids showed marked motility of mucosalis organisms incubated at 37°C but not those at 22° or 41°C.

d. Comment.

Although it is not clear why bacterial attachment was most pronounced in cultures incubated at 37°C it is possible that the effect is directly related to the active motility of the organism at this temperature.

F. ADHESIVE PROPERTIES OF KILLED C. SPUTORUM SS MUCOSALIS.

a. Introduction.

The precise nature of the attachment of C. sputorum ss mucosalis to cell cultures is not fully understood but the available evidence suggests that bacterial motility, together with the presence of appropriate receptors on the surfaces of cells and/or bacteria, play an important role in the mechanisms involved. In this connection it should be noted that several workers including Kihlstrom and Edebo (1976); Fader et al., (1979) and Yokomizo and Shimizu (1979), reported that killed bacteria possess poor adhesive properties and, for this reason, it was decided to investigate the adhesive properties of killed mucosalis.

b. Design of experiment.

i. Preparation of formol-saline killed suspensions of mucosalis.

A 24 hr-old surface growth of mucosalis strain 253/72 on a CBA slope was harvested in 10 ml of 0.25% formol-saline and the suspension incubated overnight at 37°C. The formol-saline was removed by centrifuging the bacterial suspension in three changes of sterile normal saline at 6000 x g for 15 min. The bacterial deposit was then resuspended in 10 ml of prewarmed (37°C) MEM containing 5% calf serum.

ii. Preparation of air-killed suspensions of mucosalis.

A 24 hr-old slope culture of mucosalis strain 253/72 was opened and the atmosphere ignited to remove the hydrogen microaerophilic atmosphere. The cap was replaced and the slope was incubated aerobically for 24 hr at 37°C. The air-killed bacteria were then harvested in 10 ml of prewarmed (37°C) MEM containing 5% calf serum.

iii. Preparation of suspensions of viable mucosalis organisms.

These were prepared by the standard procedure described in Chapter IV, p.95. In brief, a 24 hr-old CBA surface growth of mucosalis strain 253/72 was suspended in 10 ml of prewarmed (37°C) MEM containing 5% calf serum.

iv. Cell culture inoculation procedures.

Inoculation of cell cultures was initiated by replacing the supernatant fluids of one-day-old preformed coverslip monolayers of PK, PK_{pi}, BK and BK_{pi} with 1 ml of formol-saline-killed, air-killed or viable bacterial suspensions. Coverslips incubated at 37°C were removed at hourly intervals for the first 8 hr, rinsed in several changes of PBS to remove unattached bacteria, fixed in methanol and stained with Giemsa.

c. Results.

Significant differences were apparent in the degree of attachment displayed by the different inocula. As was expected, viable bacteria attached in large numbers and appeared to be mainly concentrated around the periphery of the cell. This may not represent the true picture since those adhering to the upper surface of the cell were more difficult to recognise. The picture obtained with air-killed bacteria was, however, different in that the organisms were uniformly dispersed over the cell surface and showed no particular preference in stained preparations for the periphery of the cell. Moreover, air-killed bacteria, unlike viable organisms, did not show increased adherence to PK_{pi} or BK_{pi} cells and attachment was similar to that on PK or BK cells. Surprisingly, the degree of attachment of air-killed bacteria remained unchanged during the period of this experiment (8hr) and additional tests confirmed that these organisms were demonstrable on the cell surface

for several days (7 days). There was no appreciable increase in the adherence of air-killed bacteria after continued incubation and almost all of the infected cells showed small numbers of attached bacteria which did not exceed 10 per cell. Thus, the adhesive property of air-killed bacteria appears to differ from that of viable organisms which is rather 'transitory' and lasts for only 10 to 12 hr post-inoculation. In contrast, formol-saline killed bacteria failed to attach to PK, PK_{pi}, BK and BK_{pi} cells during the period of this experiment.

d. Comment.

Differences in the ability of viable, formol-saline inactivated or air-killed mucosalis to attach to cell cultures emphasizes the complex nature of the adhesion phenomenon. At this stage, very little is known about the nature of bacterial and host cell surface adhesins (receptors) involved in the attachment of mucosalis, but the available evidence suggests that:

i. active motility of mucosalis appears to be a major factor in promoting bacterial attachment,

ii. inactivated mucosalis organisms, which are no longer motile, seem to possess poor or no adhesive properties,

iii. aerobic cultivation of mucosalis does not destroy completely the organism's ability to attach to cell surfaces and this clearly implies that adhesion is not entirely dependent on bacterial motility. It is possible, therefore, that specific receptors on

the surfaces of bacterial cells play an important role in the adhesion process,

iv. formol-saline treatment of mucosalis produces changes in bacterial surfaces which renders them non-adhesive. This may be due to a number of factors such as denaturization of cell surface proteins.

G. EFFECT OF SPECIFIC ANTISERUM ON THE ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO CELL CULTURES.

a. Introduction.

Several workers have studied the effects of environmental, host and bacterial factors on the attachment of pathogenic bacteria to cell surfaces and it has been shown that specific antiserum inhibits this reaction (Wilson and Hohmann, 1974; Fader et al., 1979; Yokomizo and Shimizu, 1979). This is probably achieved by immobilization, agglutination or lysis of the bacterial cells or by antiserum blocking specific receptors on the surfaces of either the host or bacterial cells. An examination of C. sputorum ss mucosalis attachment was therefore carried out in cell cultures pretreated with specific antiserum.

b. Design of experiment.

One-day-old coverslip cultures of PK, PK_{pi}, BK, BK_{pi} cells were refed with MEM incorporating 5% heat inactivated calf serum and 1% rabbit anti-mucosalis (strain 253/72) serum (titre $^{1}/2560$) prepared by the method described in Chapter II. After 2 hr' incubation at 37°C, the pre-treated cells were rinsed in two changes of PBS to remove

traces of serum, and overlaid with 1 ml of a suspension of mucosalis strain 253/72 and incubated at 37°C. A number of appropriate coverslip preparations, untreated with mucosalis antiserum, were inoculated with 1 ml of bacterial suspension and used as controls.

In a second series of experiments, the supernatant medium of one-day-old coverslip monolayers of PK, PK_{pi}, BK and BK_{pi} cells was replaced with 1 ml of a suspension of mucosalis strain 253/72 prepared as described above except that 1% specific antiserum was added to the bacterial suspension immediately prior to inoculation and incubation at 37°C. In all these cases, coverslips were removed at 2, 4, 6, 8 and 10 hr post-inoculation and examined after staining them by Giemsa's method.

c. Results.

Examination of stained preparations showed that PK, PK_{pi}, BK and BK_{pi} cells exposed to mucosalis antiserum prior to inoculation, or at the time of inoculation, failed to attach mucosalis organisms. Untreated control cultures showed good attachment of this organism to all of the cell types during the period of this experiment.

d. Comment.

It is emphasized that the C. sputorum ss mucosalis antiserum was prepared against whole, live bacterial cells and probably contains antibodies to most of the bacterial surface components including flagella. Nevertheless, the ability of this antiserum to prevent attachment is further evidence of the specific nature of mucosalis attachment, although the mechanisms involved are not understood.

H. CHARACTERISATION OF CELL SURFACE RECEPTORS INVOLVED IN THE ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO CELL CULTURES.

a. Introduction.

Lectins have been used by a number of workers in their investigations of the architecture of cell surfaces and particular attention has been paid to the localization and function of carbohydrates at this site. The role of lectins and their interactions with host cell surfaces have been reviewed by Nicolson (1974). In Virology, lectins have been successfully used to characterize the cellular surface changes observed during virus infections and to show, in some cases at least, that binding of lectins inhibits assembly and release of viruses from infected cells. It has also been shown that binding of lectins to BHK cells inhibits cell fusion induced by Simian Virus (SV 5). Furthermore some lectins have been found to bind and agglutinate selectively cells transformed by oncogenic viruses or by chemical carcinogens, thus providing useful information on the surface properties of transformed cells in comparison with their untransformed counterparts (Burger, 1973; Nicolson, 1974).

However, parallel applications of lectins in Bacteriology are unknown, although recently their value is becoming increasingly recognised in analysis of bacterial cell surfaces. Lectins have been found to react specifically with sugar residues or moieties on the bacterial cell surface, often resulting in their agglutination. Examples include lactobacilli (Fuller, 1975; Fuller and

Brooker, 1980); streptococci (Kohler and Prokop, 1967a, b; Ottensooser *et al.*, 1974); *N. gonorrhoeae* (Schaefer *et al.*, 1979); mycoplasmas (Schiefer *et al.*, 1974) and chlamydias (Levy, 1979). Inhibition of *C. psittaci* attachment to L-cells treated with wheat germ lectin has also been reported (Levy, 1979).

In the present study, an attempt was made to explore the nature of the host cell surface receptors involved in the attachment of *mucosalis*. Several types of lectins are available but Concanavalin-A (Con-A) was prefered as it has been extensively used in characterising the surface properties of 'transformed' cells and because of growing evidence that 'mannose-like' carbohydrate receptors are involved in the attachment of *E. coli* to cell surfaces *in vitro*.

b. *Design of experiment*.

One-day-old coverslip preparations of PK_{pi} and BK cells, rinsed in 2 changes of PBS, were flooded with a suspension of fluorescein-labelled Con-A at 50 µg/ml protein (Calbiochem-Behring Corp., La Jolla, Calif.) and incubated in a moist atmosphere in Petri dishes at 37°C for 30 min. After thorough rinsing in several changes of PBS to remove unbound Con-A, the coverslip preparations were placed in fresh test tubes and bacterial attachment was initiated by overlaying the monolayers with 1 ml of *mucosalis* suspension in MEM. These, together with a number of uninfected Con-A treated coverslips as controls were incubated for 1,2 and 3 hr at 37°C, rinsed 3 times to remove unattached

bacteria, mounted in PBS and examined under a U-V microscope for binding of Con-A.

In extension of this experiment, coverslips of PK_{pi} and BK cells, initially exposed to mucosalis for 1, 2 and 3 hr at 37°C, were subsequently treated with Con-A and similarly examined. The specificity of Con-A blocking of mucosalis receptor sites on the host cell surfaces was checked by pretreating monolayers with the Con-A antagonist, methyl α-D mannopyranoside (0.5%), incorporated in MEM for 1 hr at 37°C and exposing washed monolayers to either Con-A or mucosalis as described above. Coverslips were also examined after staining by Giemsa's method.

c. Results.

TheU-V microscopic examination of PK_{pi} and BK cells exposed to Con-A showed extensive fluorescence on the surface of cells indicating specific binding of Con-A to host cell receptors. Pre-treatment of cells with methyl α-D-mannopyranoside completely inhibited the binding of Con-A, resulting in the absence of fluorescence.

However, compared with uninfected Con-A treated controls, monolayers preincubated with mucosalis and subsequently treated with Con-A, or vice-versa, invariably showed moderate fluorescence suggesting that only limited binding of the lectin had taken place. Corresponding monolayers stained by Giemsa showed evidence of a reduction in bacterial attachment which might account for the reduced fluorescence observed above. There was no appreciable difference in the Con-A binding ability of PK_{pi}

and BK cells nor in the degrees of mucosalis attachment to cells pretreated with the lectin.

A series of supplementary experiments showed that increased concentration and prolonged incubation with Con-A, or pretreatment and maintenance of coverslips in MEM supplemented with Con-A antagonist, methyl α-D-mannopyranoside, did not prevent the attachment of mucosalis to PK_{pi} and BK cells.

d. Comment.

Although investigations of the blocking of cell surface receptors specific for mucosalis adhesins were limited to the use of only one lectin, i.e. Con-A, evidence was obtained and showed that i) Con-A was able to bind PK_{pi} and BK cells, ii) attachment of mucosalis to the surfaces of these cell types greatly reduced subsequent binding of Con-A, and iii) pretreatment with either Con-A or mannopyranoside reduced but failed to completely block the attachment of mucosalis. This information points to the possible presence of multiple surface receptors for mucosalis on the host-cells.

DISCUSSION

In the present study, the ability of mucosalis organisms to attach to a wide range of cell cultures, including primary pig kidney cells (PPK) is an important observation considering that bacterial attachment to host cells is now generally accepted as being the first step in the development of an infectious process (Jones

1977; Smith, 1977; Arbuthnott and Smyth, 1979).

It is also of interest that all four strains of mucosalis examined in this series of experiments were not only capable of attachment to permissive cell types but produced a similar pattern of 'transitory' bacterial attachment which reached a maximum around 4 to 6 hr but lasted for only 10 to 12 hr post-inoculation. Although no precise reason could be given to explain the 'transitory nature' of the adhesion process, the available evidence suggests that the termination of attachment of free extracellular organisms might be associated with the rapid death of bacteria in an unfavourable gaseous environment since preliminary observations showed that mucosalis is rapidly killed, in 12 hr, when incubated in a cell-free aerobic atmosphere.

The inability of mucosalis organisms derived from 'parasitic growth' to attach to cell cultures from 24 hr onwards, despite the presence of large numbers of bacteria in the supernatant fluids,is also difficult to explain. In future cell-culture studies, further consideration should be given to the adhesive properties of bacteria produced by 'parasitic growth' with particular reference to the effects of bacterial metabolites on the host-cell surface receptors.

It is evident from the present findings that bacterial motility plays an important role in the degree of attachment achieved by mucosalis and that factors affecting bacterial motility will greatly reduce bacterial attachment. There is also evidence that the surface

components of the bacterial cell play a significant part in the process of attachment since treatment of mucosalis with formol-saline prevented adhesion whereas the adverse effects of aerobic incubation did not destroy the surface components completely and permitted limited bacterial attachment.

The nature of the host-cell receptors involved in mucosalis attachment is not fully understood although, experiments using Con-A binding to host cell pointed to the possible existence of multiple surface determinants specific for mucosalis.

The inability of mucosalis organisms to attach to CEF, Vero and $LLCMK_2$ cells on primary infection and to the 'altered behaviour' of Vero and $LLCMK_2$ cells to re-infection when they showed limited bacterial attachment suggest that specific receptors on these cells are small in number, become modified by exposure to infection or, in the case of CEF, are absent.

CHAPTER VII

CHAPTER VII

DEMONSTRATION OF INTRACELLULAR C. SPUTORUM SS MUCOSALIS IN INFECTED CELL CULTURES.

GENERAL INTRODUCTION.

A review of published work on the growth and development of pathogenic bacteria in cell cultures (Chapter I) indicates that, in most cases, the exposure of cells to infection results in phagocytosis of the organism with or without subsequent intracellular multiplication (Giannella et al., 1973; Waitkins and Flynn, 1973; Bovallius and Nilsson, 1975; Kihlstrom, 1977; Egwu and Eveland, 1979; Brunius, 1980).

Several methods including light microscopy, viable counts, immunofluorescence and electron microscopy, have been found useful to demonstrate the presence of intracellular bacteria although these methods, when used individually, do not necessarily provide conclusive evidence of the location of the organism. However, an overall picture of the intracellular fate of pathogenic bacteria in the infected cells may emerge when all of these procedures are employed.

In the present investigation it has been shown that C. sputorum ss mucosalis is capable of attachment to a wide range of both primary and established cell cultures, and viable bacterial counts on infected cell cultures strongly suggest the possibility of intracellular multiplication of this organism. The following work was

undertaken by light microscopy in an attempt to follow the fate of the bacteria after initial attachment to cell surfaces.

I. A. DEMONSTRATION OF INTRACELLULAR C. SPUTORUM SS MUCOSALIS IN MONOLAYER CULTURES.

a. Introduction.

In view of the observation that C. sputorum ss mucosalis attached in large numbers to both trypsinized cells and preformed monolayers of a wide range of cell cultures (Chapter VI), these cultures were examined for their ability to phagocytose this organism. The initial examinations were straight forward and involved the use of light microscopy in attempts to demonstrate the intracellular location of mucosalis in cells inoculated before and after the monolayers had formed.

b. Design of experiment.

The techniques of infecting suspensions of trypsinized cells and 24 hr-old preformed monolayers have already been described in Chapter IV. The cell cultures employed were primary pig kidney (PPK), chicken embryo fibroblasts (CEF) and lines of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, DK, BHK, HeLa, Vero and $LLCMK_2$ cells. Each of these cell cultures, in the form of coverslip preparations, were exposed separately to mucosalis strains 253/72, 982/76, 512/77 and 1075/78. 'Flying coverslips' of infected and uninfected monolayers were incubated at 37°C, and collected daily for 2 weeks and rinsed in several changes of warm PBS to

remove unattached bacteria. Methanol-fixed coverslips were stained with Giemsa and examined by light microscopy.

It should be emphasized at this stage, that particular attention was paid in this present experiment to the intracellular fate of mucosalis organisms in the infected cell cultures. Although the cytopathic changes observed are described in detail in Chapter VIII, a brief description of the cellular abnormalities in relation to the intracellular location of mucosalis is included in the text of this chapter.

d. Results.

Demonstration of mucosalis in infected monolayers.

i. One day post-inoculation.

At 24 hr, almost all the mucosalis organisms were present within phagosomes* and the cell surfaces remained free of attached bacteria. Phagocytosed bacteria stained reasonably well and retained their usual vibrioid morphology (Fig. 9A).

---

*The term 'phagosome' is used here to describe the artificially-induced cytoplasmic vacuoles associated with the phagocytic activity of the cell and does not include the cytoplasmic vacuoles which are a normal feature of many cell cultures. The only way however that these vacuoles can be differentiated is by the presence of bacteria contained within the former.

PK and PK_{pi} cells showed a large number of intracellular bacteria in most of the infected cells compared with moderate numbers in many of the PPK, BK, BK_{pi}, OK_{pi}, DK, BHK and HeLa cells. At this stage, the number of intracellular bacteria ranged from a few to 20 or more per cell, but attempts to quantify their numbers in different cell types was greatly hampered by the clumping of organisms in the phagosomes so that individual bacteria could not be resolved in Giemsa stained preparations.

Intracellular bacteria were not detected in stained monolayers of primary chicken embryo fibroblasts (CEF), Vero or $LLCMK_2$ cells but this was not unexpected since none of these cell types permitted the attachment of mucosalis to cell surfaces (Chapter VI).

ii. Two-days post-inoculation.

By the 2nd day, bacteria were occasionally seen in phagosomes but regularly occurred elsewhere in the cytoplasm, and this intracellular migration of bacteria was associated with a reduction in the number and size of the phagosomes. The bacteria had largely lost their typical vibrioid morphology and the majority appeared 'coccoid' in form. At this stage bacteria were observed in greatly reduced numbers in all cell types examined, and stained very faintly. There was early evidence of 'rounding up' of cells in PK cultures and of cell fusion in PK_{pi} cells, while other cell types remained apparently normal. PPK cells showed evidence of increased cytoplasmic vacuolation many of which did not contain

mucosalis organisms.

iii. Three days post-inoculation.

An absence of detectable intracellular bacterial forms in Giemsa stained preparations was characteristic of all cell cultures examined at this period. The majority of infected PK cells had rounded up and were beginning to detach from the glass whereas PPK and PK_{pi} cells showed extensive cell fusion with polykaryon formation.

iv. Four days post-inoculation.

After 4 days post-inoculation cell types infected with mucosalis could be divided into two major groups based on the fate of intracellular bacteria and on the changes taking place in the cells. Pig kidney cell cultures (PPK, PK and PK_{pi} cells) formed one group in which degenerative changes were extensive, culminating in the total destruction of cell sheets in less than 5 days. These cells did not show any evidence of the reappearance of intracellular bacteria forms. The other group included BK, BK_{pi}, OK_{pi}, BHK and HeLa cell cultures which appeared to be more resistant to infection and, despite a ballooning effect, infected cell sheets remained attached to the glass for at least 2 weeks. In addition some of these cells showed poorly visible intracytoplasmic granules which appeared to have replaced intracellular bacterial forms seen at earlier stages in the infection (Fig.10A). Marked swelling of the cytoplasm was easily recognisable in the majority of the infected cells. By contrast, DK cells remained apparently normal and did not show intracellular bacteria.

v. Five days onwards post-inoculation.

The unusual intracytoplasmic granules in the 2nd group of less permissive cells had slightly increased in size and were visible as deeply stained 'coccoid' and short rod-forms against a paler staining background of host cell cytoplasm. These intracellular bodies appeared to develop during further incubation until by the end of 6 to 7 days post-inoculation, they appeared to have undergone a fundamental change into predominantly, 'novel', filamentous forms which were often aggregated into clumps and had migrated towards the periphery of the affected cells. These filamentous structures were of variable length, thinner than mucosalis and irregularly wavy in form. These intracellular 'novel' forms persisted without further morphological change and their presence was invariably associated with massive enlargement of the cell cytoplasm which appeared thin and featureless. Similar intracellular filamentous forms were not detected in pig kidney cell cultures. (Figs. 10B-C).

Although 'novel' bacterial forms could be detected in the thin and featureless cytoplasms of the enlarged infected cells for upto 2 weeks, they were not present in the apparently normal cells which accounted for less than 30% of the infected cell population.

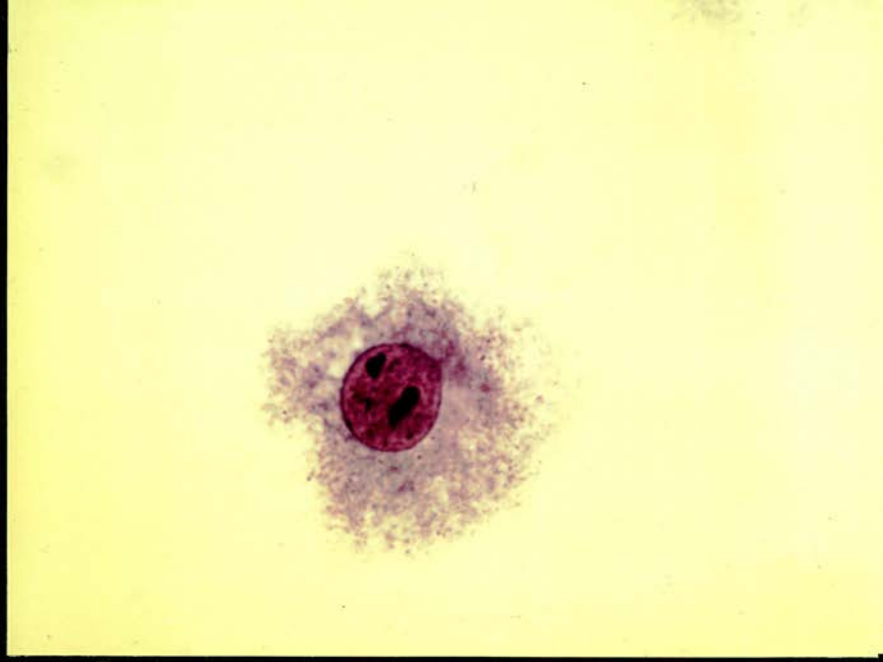
However, similar cellular changes, and the presence of intracellular bacterial forms, were not a characteristic feature of DK cells although a slight 'ballooning' of cells was evident in about 30 to 40% of the cell population at 2 weeks post-inoculation.

Fig. 9A

A single cell in a 24 hr-old monolayer culture of PK_{pi} cells infected with C. sputorum ss mucosalis at the time of seeding. Notice the absence of bacterial attachment to the cell surface and the presence of large numbers of organisms within the cytoplasm.
Giemsa X 1500.

Fig. 9B

Uninfected 24 hr-old PK_{pi} cell control.
Giemsa X 1500.



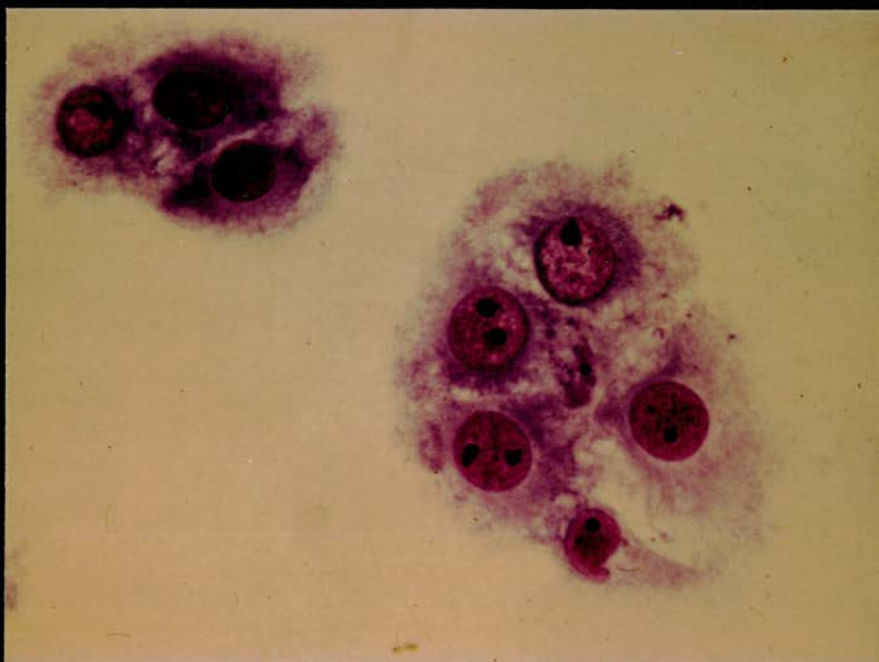


Fig. 10A

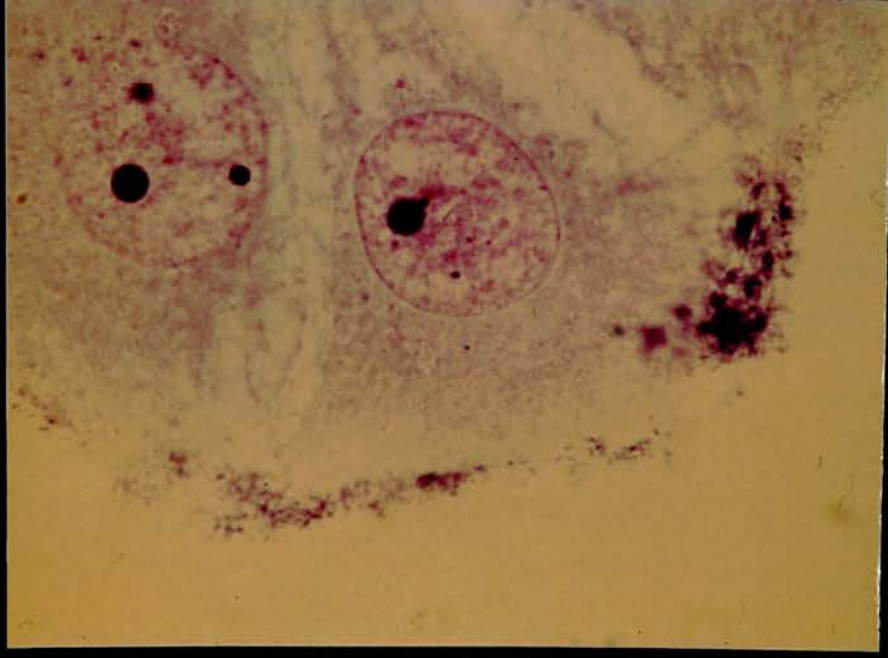
Giemsa stained coverslip preparation of BK cells infected with C. sputorum ss mucosalis for 5 days. Notice the presence of faintly staining intracellular organisms (arrows) and their absence from some cells. X 1500.

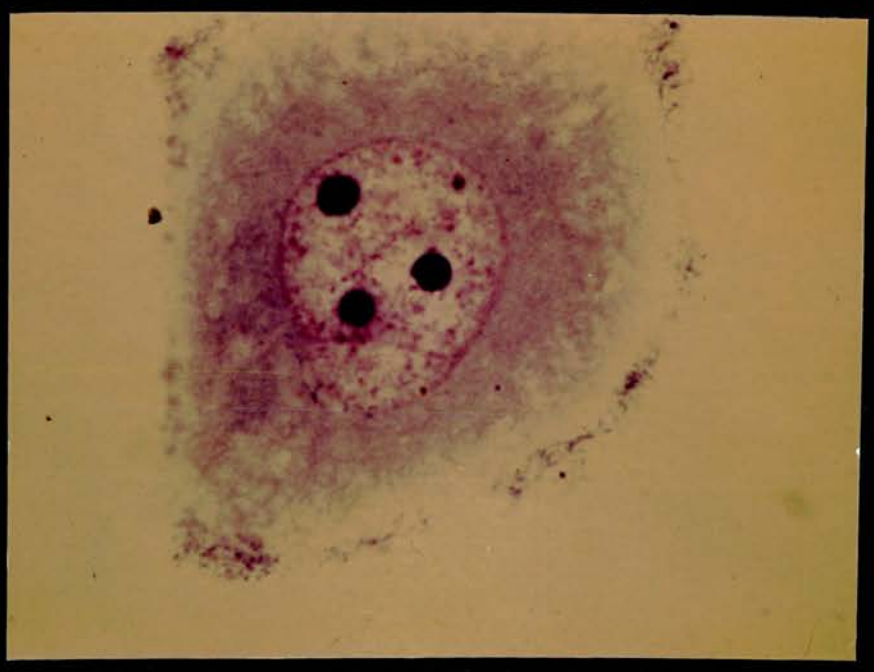
Fig. 10B

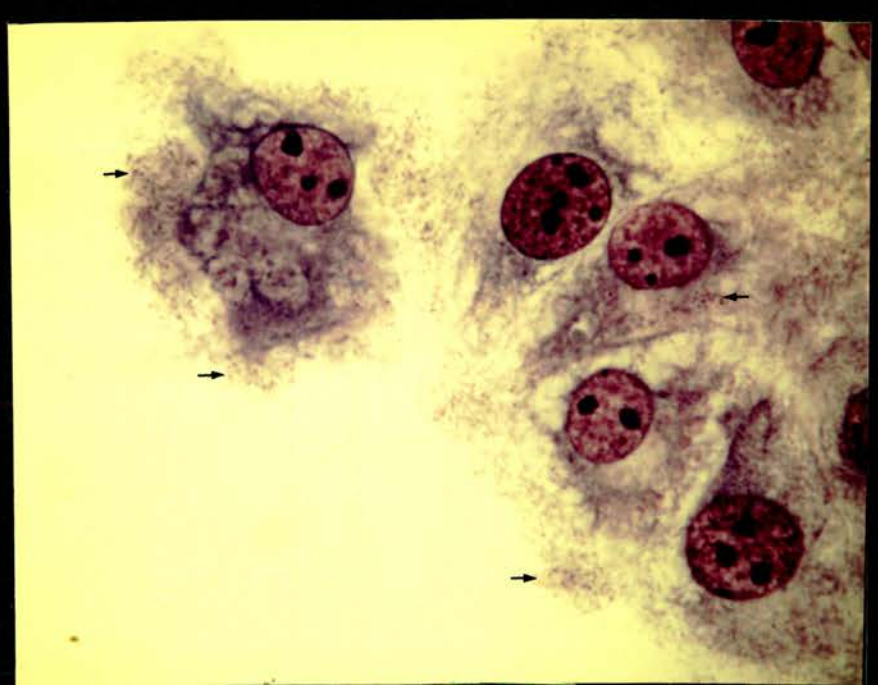
Ten-day-old BK cells infected with C. sputorum ss mucosalis at the time of seeding. Note the thin, featureless, markedly enlarged cytoplasm and the accumulation of numerous, novel bacterial forms at the periphery of the cell.
Giemsa X 160.

Fig. 10C

A similar preparation to that of Fig. 10B, showing deeply staining 'novel forms' of mucosalis at the periphery of the cells.
Giemsa X 160.







Infection of cells before or after the monolayer had formed produced no appreciable difference in the ability of 'permissive' cells to phagocytose each of the four mucosalis strains and the intracellular fate of all these strains was similar. By contrast, CEF, Vero and $LLCMK_2$ cells failed to show any demonstrable intracellular bacteria when stained by Giemsa's method.

In all these cases, uninfected cultures remained healthy and did not show structures resembling intracellular mucosalis forms or other cellular abnormalities (Fig. 9B).

d. Comment.

The demonstration of intracellular mucosalis organisms in a wide range of cell cultures is of interest and appears to indicate that:

i. bacterial attachment is a prerequisite for phagocytosis as cell types that failed to attach mucosalis were invariably negative for demonstrable intracellular bacteria.

ii. phagocytic activity of the 'permissive' cell cultures was restricted to the first 24 hr post-inoculation and, despite the presence of viable extracellular bacteria, there was no clear evidence of fresh attachment nor phagocytosis of this organism after this period. In fact, the phagocytosed bacteria tended to disappear from the cytoplasm after 24 hr.

iii. Intracytoplasmic organisms lost their typical vibrioid morphology and, changed to 'coccoid forms' which, at first, could be recognised with ease but tended to disappear on further incubation. Some cell types appeared to be less susceptible to the destructive effects of bacterial infection (BK, BK_{pi}, OK_{pi}, BHK, HeLa and, possibly, DK cells) and permitted intracellular development of 'novel' bacterial filamentous forms 6 to 7 days post-inoculation. In contrast, pig kidney cells cultures were destroyed by this time and it was not possible, therefore, for filamentous forms to develop.

iv. The long, filamentous, 'novel' bacterial forms were typical in their own way, and were morphologically distinct from either the commonly encountered mycoplasmas or from mucosalis organisms grown in routine bacteriological media. At this stage in the investigation the relationship of these 'novel' forms to mucosalis was not clear.

It is emphasized that these observations must be confirmed by more refined and accurate procedures, such as immunofluorescence and electron microscopy, before the role of these forms in the developmental cycle of mucosalis can be established. The technique of light microscopic examination of stained monolayers is generally considered to be one of the least useful methods

of evaluating intracellular events although the overall usefulness of this simple procedure cannot be ignored.

B. DEMONSTRATION OF INTRACELLULAR C. SPUTORUM SS MUCOSALIS IN SUSPENSION CELL CULTURES.

a. Introduction.

Infection of preformed monolayer cultures with C. sputorum ss mucosalis provided useful information on the intracellular location of this organism, but the procedure was less useful for recognising the early events of attachment and phagocytosis.

It was decided, therefore, to confirm the light microscopy findings reported in the previous experiment by similar studies using suspension cell cultures wherein cells are separated from one another and the interaction of mucosalis with individual cells can be monitored more effectively.

b. Design of experiment.

Monolayers of PK, PK_{pi}, and BK cells were detached from the glass with STV and 4 ml of trypsinized cell suspension, containing 5×10^5 cells/ml, were added to 40 ml of warm ($37^{\circ}C$) suspension medium S-MEM (Gibco Biocult, Scotland) in a Bellco-spinner flask (100 ml capacity). The completed medium contained Earle's salts, 25 mM HEPES buffer, 0.2 mM L-glutamine and 10% heat inactivated calf serum with the addition of 0.3% methyl cellulose to prevent clumping of the suspended cells.

Infection was initiated by adding 10 ml of a suspension of mucosalis strain 253/72 in S-MEM (approximately 5 x 10^6 bacteria/ml) to the cell suspension. The spinner flask containing cells and bacteria was incubated at 37°C on a magnetic stirrer to keep the cells in suspension. Each day the cell cultures were refed by replacing 10 - 15 ml of the cell ~~suspension~~ supernatant with fresh prewarmed S-MEM, and incubation was continued.

A sample of the cell suspension was withdrawn at 2, 4, 8, 10, 12, 24, 48 and 72 hr post-inoculation and the cells were spun on to a clean glass slide in a cytocentrifuge (Shandon Co. England) at 1000 x g for 3 to 5 min. These cell smears were fixed in acetone, stained with Giemsa and examined in a light microscope.

c. Results.

Cell suspensions collected after 2 hr of incubation showed that no fewer than 70 to 80% of the cells permitted bacterial attachment and some had as many as 10 or more bacteria adhering to their surface. At this early stage of infection, it was evident that PK and PK_{pi} cells were capable of attaching larger numbers of bacteria than were BK cells (Fig. 11A).

Cytocentrifuge preparations examined at 4 hr indicated bacterial attachment to almost all cells and this was particularly marked in PK_{pi} cells where the cells were covered by a matt of tightly packed bacteria. By this time, the rounded trypsinized cells were beginning to assume their normal epithelial morphology and close examination showed the presence of small clumps of a few

bacteria within cytoplasmic vacuoles (phagosomes) but not in the cytoplasm itself. The majority of infected cells, compared with controls, contained a large number of cytoplasmic vacuoles, which was, perhaps, an indication of their phagocytic activity. In addition to the presence of mucosalis within the vacuoles, the preparations also showed a large number of cells in the process of englufing bacteria .

Cell preparations examined between 4 to 12 hr showed a gradual decrease in the number of attached bacteria after 6 hr and only a few organisms could be detected on the cell surfaces by the end of 12 hr of incubation. The presence of only occasional bacteria on the cell surfaces seemed, once again, to indicate the absence of bacterial attachment at this stage (Fig.11B).

The phagocytic activity of the cells appeared to be maximal around 4 to 8 hr after infection and many phagosomes contained upto 10 to 15 organisms, although about 20% of these vacuoles remained free of any demonstrable organisms. In general, the infected cells showed increased vacuolation compared with the controls.

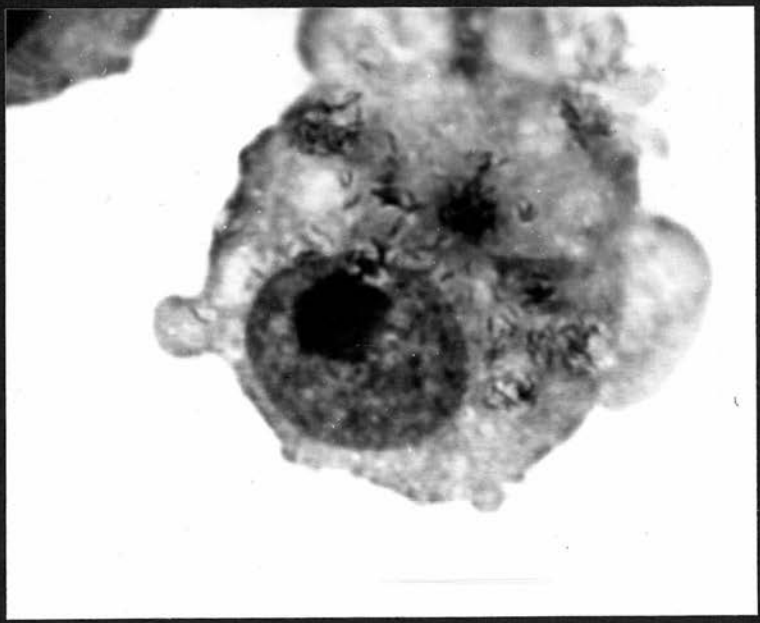
Phagocytosis of mucosalis was invariably followed by migration of these organisms into the cytoplasm from the phagosomes. By 12 hr, a number of cells showed organisms apparently free in the cytoplasm although the majority of the bacteria remained in the phagosomes. Apart from increased cytoplasmic vacuolation the infected cells appeared normal and phagocytosed bacteria showed their usual morphology and stained well at this stage.

Fig. 11A

A cyto-centrifuged preparation of a suspension of PK_{pi} cells inoculated with C. sputorum ss mucosalis. After 2 hr incubation a large number of organisms are attached to the cell surfaces. Notice the unattached extracellular bacteria. Giemsa X 2000.

Fig. 11B

Eight hours post-inoculation, the periphery of the cell is free of attached bacteria but clusters of organisms appear to be located within the cytoplasm. Giemsa X 2000.



Suspension cultures examined 24 hr post-inoculation showed increased numbers of bacteria in the cytoplasm, decreased cytoplasmic vacuolation and complete absence of adhering bacteria; but the intracytoplasmic bacteria stained weakly and were less characteristic in their morphology. Occasional organisms were still visible in the phagosomes and were usually 'coccoid' rather than vibrioid in shape. These findings were consistent in all the three cell types examined.

Also at this stage (24 hr) many of the infected cells showed early degenerative changes and their nuclei stained deeply. Despite constant stirring of the cell suspensions, large deposits of cells occurred at the bottom of the spinner flask and vital staining with neutral red indicated that over 50% of the cells had died. This situation did not improve after replacing upto a third of the volume with fresh S-MEM.

Cell suspensions of PK, PK_{pi} and BK cells examined at 48 hr post-inoculation indicated complete cellular destruction with heavy deposits of dead cells in the spinner flask and a clear supernatant medium. Vital staining with neutral red indicated that less than 10% of the cells were viable in the culture.

Uninfected cultures of PK, PK_{pi} and BK cells, like their mucosalis infected counterparts, did not adapt readily to growth in suspended cultures and were completely destroyed in 2 to 3 days post-inoculation. Further attempts to adapt these or other cell types to

grow in suspension were not made since the results of this experiment adequately confirmed the initial events of bacterial attachment and subsequent phagocytosis by cultured cells.

d. Comment.

The use of suspension cultures to examine the process of bacterial attachment and subsequent phagocytosis proved to be satisfactory in differentiating these early events. Unlike preformed monolayers, suspension cultures showed uniform attachment of mucosalis organisms to cell surfaces, and such adherence did not appear to be restricted to the periphery of the cells. The phagocytic activity of PK, PK_{pi} and BK cells was particularly impressive and the process of engulfing of bacteria was clearly visible during the early phase of infection. The impression gained was that this active engulfment by the cell was possibly the main method by which these bacteria gain entry to the cell. The results of this experiment again confirmed the 'transitory' nature of the attachment of mucosalis organisms. Further studies involving cell cultures adapted to growth in suspensions should be useful to understand the fate of this organism in its intracellular location, but this was not possible in this particular experiment due to early death of the infected cells.

C. DEMONSTRATION OF INTRACELLULAR C. SPUTORUM SS MUCOSALIS IN CELL CULTURES BY IMMUNOFLUORESCENCE STAINING.

a. Introduction.

Immunofluorescence staining has been used by several workers for the demonstration and assessment of intracellular multiplication of bacteria in infected cell cultures (Bovallius and Nilsson, 1975; Kihlstrom, 1977; Brunius, 1980). Despite its high degree of specificity the technique, as commonly employed, fails to differentiate between intracellular bacteria and those located on cell surfaces, nor does it clearly distinguish between viable or dead bacteria and degraded bacterial antigens.

Although a variety of techniques have been devised to evaluate the association of bacteria with cell cultures, there appears to be no simple method for distinguishing between intra- and extracellular organisms. Workers have, in the past, relied on the inability of antiserum to penetrate the outer membrane of unfixed cells to demonstrate bacteria bound to cell surfaces, and on pretreatment of infected cells with acetone (which is believed to facilitate the passage of antibodies through the cell membranes) to recognise intracellular organisms (Nairn, 1976).

In recent years, medical virologists have obtained evidence that prefixation of cells with formaldehyde prevents the penetration of antibodies through cell

membranes and such a procedure has been found to be particularly useful in locating viral antigens on cell surfaces (Fraser, 1976).

In the present investigation the evidence so far obtained from viable counts of bacteria recovered after lysis of infected cells has indicated that intracellular multiplication of mucosalis may take place. This finding was further substantiated by light microscopic observations which indicated that certain types of infected cells consistently contained large numbers of 'novel' bacterial forms as aggregates within the cytoplasm.

In view of these observations, confirmation was sought as to the ability of this organism to multiply intracellularly, and to establish whether or not these 'novel' bacterial forms are, in fact, mucosalis organisms. For this purpose indirect immunofluorescence staining of infected cell cultures with antiserum specific for mucosalis was employed.

b. Design of experiment.

Two separate experiments were carried out in order to examine i) the initial events of bacterial attachment and phagocytosis, and ii) the subsequent fate of intracellular organisms in PK_{pi} and BK cells.

i. Attachment of mucosalis to cell surfaces.

The method of infecting trypsinized cell suspension of PK_{pi} and BK cells with strain 253/72 has already been described on page 96. Infected and uninfected coverslip preparations, incubated at 37°C, were collected at 2, 4, 6, 8, 10, 12 and 24 hr post-inoculation and then rinsed

in several changes of PBS to remove unattached bacteria. Coverslips were then fixed for either 10 min in ice-cold acetone or for 30 min in 10% neutral formaldehyde (Fraser, 1976). Immunofluorescence staining of these prefixed monolayers was carried out using rabbit anti-C. sputorum ss mucosalis serum (strain 253/72 - 'OH' antiserum) and FITC labelled sheep anti-rabbit immunoglobulin by the method described in Chapter II.

ii. Fate of mucosalis within infected cell cultures.

This experiment is a continuation of the above procedures, and coverslip preparations were collected during the first 4 days and on the 7th, 14th and 21st days post-inoculation. After thoroughly rinsing the monolayers in PBS to remove unattached bacteria, representative coverslips were fixed in either acetone or formaldehyde and examined by immunofluorescence staining as described above.

iii. The presence of mucosalis antigen in serially passaged BK cells.

Trypsinized cell suspensions of BK cells were inoculated with mucosalis strain 253/72 and allowed to form monolayers at 37°C in 4 oz medical flats. These and uninfected control cultures were refed routinely at weekly intervals and subcultured every three weeks. A portion of the trypsinized cell suspension obtained at each subculture was seeded on coverslips and incubated at 37°C for 2 days. The monolayers were then prepared for immunofluorescence staining as described above.

c. Results.

i. Demonstration of bacterial attachment in PK_{pi} and BK cells fixed in formaldehyde.

Stained coverslip preparations showed the presence of fluorescing bacteria attached to the cell surfaces after 2 hr and the process appeared to be progressive since large numbers of organisms could be seen in preparations obtained at 4 and 6 hr post-inoculation. However, a rapid drop in bacterial attachment was apparent in monolayers examined at 8 hr, and later preparations showed only occasional organisms attached to cell surfaces but none at 24 hr post-inoculation. At no time was there any evidence of specific fluorescence in the cytoplasm or nucleus indicating that the formaldehyde fixation procedure had largely prevented antibody from entering the cell.

PK_{pi} cells showed greater numbers of attached bacteria during the 6 hr period, compared with the moderate numbers seen on BK cells.

ii. Demonstration of bacterial attachment in PK_{pi} and BK cells fixed in acetone.

Acetone-fixed 2 hr-old coverslip cultures showed a limited number of bacteria attached to the cell surfaces in about half of the cell population but intracellular bacteria were invariably absent at this stage. Preparations examined at 4 hr showed the presence of occasional fluorescing bacteria within the cytoplasm and a further increase in the number of organisms adhering to the cell surfaces. Examination after 6 and 8 hr post-

inoculation invariably showed a large number of intracytoplasmic bacteria which usually occurred as aggregates or clumps of brightly staining material. About 60 to 70% of the cells contained fluorescing bacteria by 8 hr to 12 hr post-inoculation and only moderate numbers of organisms appeared to be bound to cell surfaces at this stage. Monolayers examined at 24 hr showed a complete absence of bacteria attaching to cell surfaces and a slight increase in the percentage of cells (70 to 80%) showing the presence of fluorescing intracellular bacteria (Fig.12A). At this stage the organisms stained brightly and retained their characteristic vibrioid morphology. They occurred singly or in groups of 2 or 3 organisms evenly distributed in the perinuclear cytoplasm and some cells contained more than 20 bacteria per cell.

Control cultures remained free of any specific fluorescence during the period of this experiment (Fig.13B).

iii. A comparative evaluation of intracellular growth of mucosalis in infected monolayers prefixed in formaldehyde or acetone.

Examination of stained monolayers of PK_{pi} and BK cells prefixed in either formaldehyde or acetone showed several interesting features. Monolayers fixed in formaldehyde consistently failed to show the presence of any specific fluorescence on the cell membranes or within the cytoplasms of infected cells from 24 hr and onwards (Fig.17A).

By comparison, acetone-fixed monolayers clearly showed brightly stained vibrioid organisms within the cytoplasm after 24 hr (Fig.12A). Examination of two-day-old monolayers indicated that the intracellular bacteria had lost their typical vibrioid morphology by this time and that the majority of the vibrios observed previously had been replaced by large numbers of small, predominantly 'coccoid' forms both in PK_{pi} and BK cells. These 'coccoid' particles were present in the perinuclear cytoplasm and tended to aggregate to form large bright areas of fluorescence, but there was no evidence of nuclear involvement. On further incubation (2 to 4 days) these 'coccoid' forms greatly increased in size and their presence was particularly noticeable within the cytoplasm of multinucleated polykaryons in PK_{pi} cultures (Fig.12B ,13A). On the 7th day post-inoculation there was almost complete loss of the cell sheet in infected PK_{pi} cultures but scanty fluorescence could still be seen in the few cells that remained attached to the glass.

Examination of BK coverslip cultures after 24 hr indicated that intracellular bacteria generally retained their 'vibrioid' morphology although a few 'coccoid forms' were sometimes seen (Fig.14A). This phase was gradually replaced after 2 to 4 days by the appearance of a 'diffuse type' of fluorescence in the infected cytoplasm. Despite the diffuse nature of the staining reaction, well defined foci of mucosalis antigens could be clearly seen in about 70% of the cell population (Fig.14B).

In general, BK cells showed comparatively fewer intracellular bacteria than PK_{pi} cells.

BK cells after 7 days incubation showed no evidence of diffuse fluorescence in any of the infected cells but, surprisingly, this change was accompanied by the appearance of large numbers of long filamentous forms which stained specifically with mucosalis-antiserum (Fig.15A). The cell surfaces remained free of attached bacteria and the intracellular forms of mucosalis appeared to undergo rapid multiplication, since monolayers examined on the 14th, and 21st day post-inoculation showed that the cytoplasm of most cells contained enormous numbers of brightly stained filamentous organisms some of which appeared spiral or vibrioid (Figs.15B,16A). The nuclei were unaffected and there was no evidence of the small 'coccoid' bodies which usually predominated in PK_{pi} cells.

Having obtained this remarkable evidence of the presence of enormous numbers of intracellular bacteria by immunofluorescence staining, it was interesting to examine, retrospectively, the corresponding coverslip cultures (21-day-old BK monolayer) stained by Giemsa's method and examined by light microscopy. The appearance of representative cells stained by the latter method is shown in Fig.17B and it is apparent that cells stained in this manner give no indication of the presence of the enormous numbers of intracellular bacteria.

iv. Persistence of mucosalis antigens in serially passaged BK cells.

Acetone-fixed coverslip cultures, unlike those fixed in formaldehyde, showed the presence of mucosalis antigens in nearly a third of the cell population, and they persisted in serially-passaged BK cells for up to 65 days. The typically long, filamentous bacterial forms, which usually predominated during the first two passages (42 days), were invariably absent at this stage and the intracellular antigens mainly consisted of small, evenly distributed 'coccoid' particles (Fig.16B). A gradual decrease in the presence of intracellular mucosalis antigen after 3 weeks was evident, and the reaction was less intense. In all cases, the acetone-fixed, uninfected controls failed to show specific intracellular fluorescence (Fig. 16C).

d. Comment.

In this immunofluorescence study of PK_{pi} and BK cells infected with mucosalis, a preliminary attempt was made to assess whether formaldehyde fixation of cell cultures prevented the passage of antiserum through the cell membranes. The 'transitory' nature of mucosalis attachment seems to provide an ideal opportunity for such an investigation.

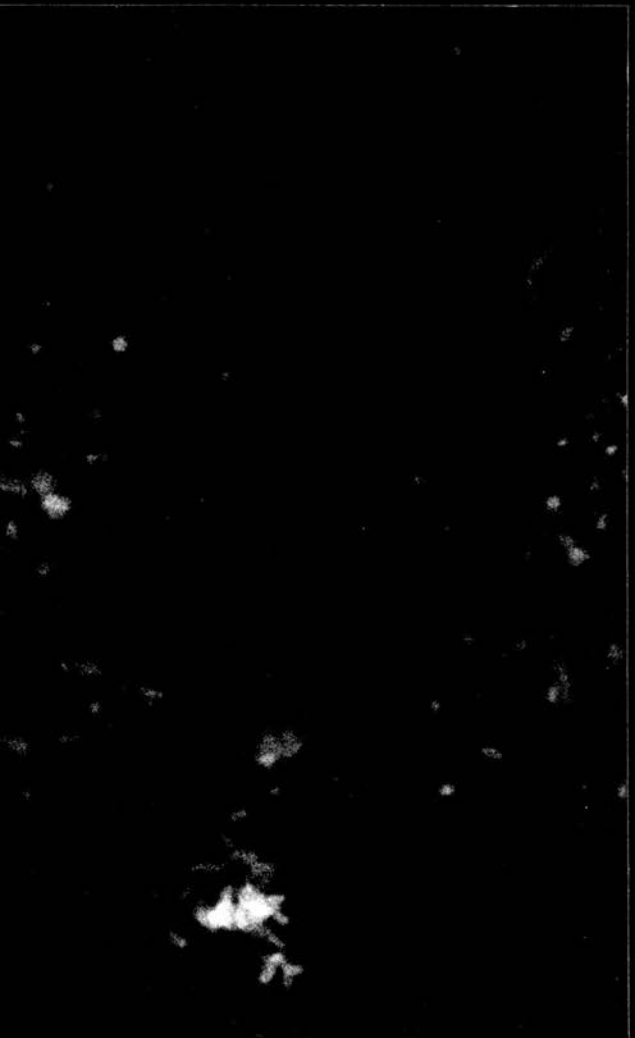
Indeed, the results showed that in formaldehyde-fixed monolayers, it is possible to demonstrate, selectively, extracellular bacterial attachment whereas the intracytoplasmic bacteria remain unstained. The inability of specific antiserum to penetrate formaldehyde-fixed

Fig. 12A

Immunofluorescence staining of PK_{pi} cells inoculated 24 hr previously with C. sputorum ss mucosalis, showing predominently coccoid and occasional vibrioid forms.
Acetone fixation X 256.

Fig. 12B

Immunofluorescence staining of PK_{pi} cells two days after infection with C. sputorum ss mucosalis. Notice the greatly increased numbers of fluorescing particles of mucosalis in the perinuclear region of the infected cell. Acetone fixation X 256.



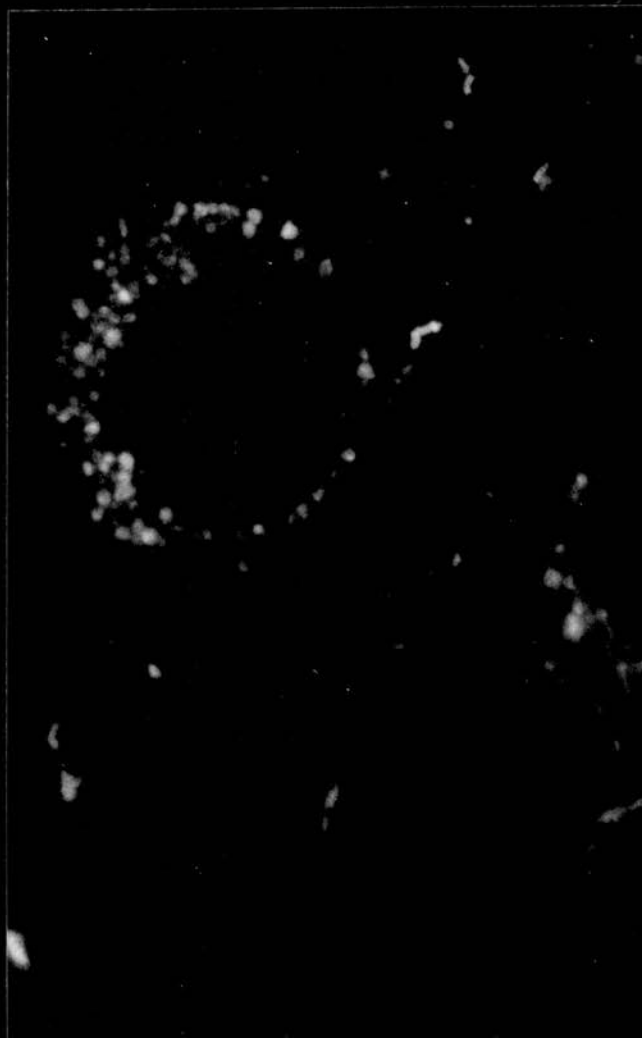


Fig. 13A

After 4 days' incubation, infected PK_{pi} cells show numerous foci of marked fluorescence indicating the presence of *mucosalis* antigen in the cytoplasm of the cells. Acetone fixation X 256.

Fig. 13B

Immunofluorescence staining of 4-day-old uninfected PK_{pi} control. Acetone fixation X 256.

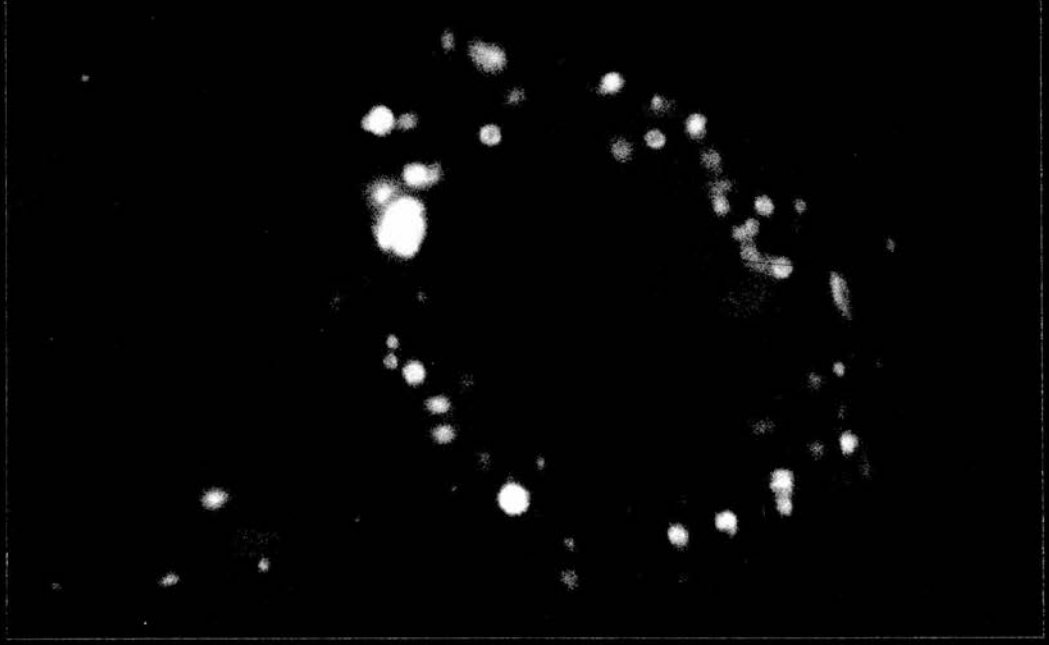
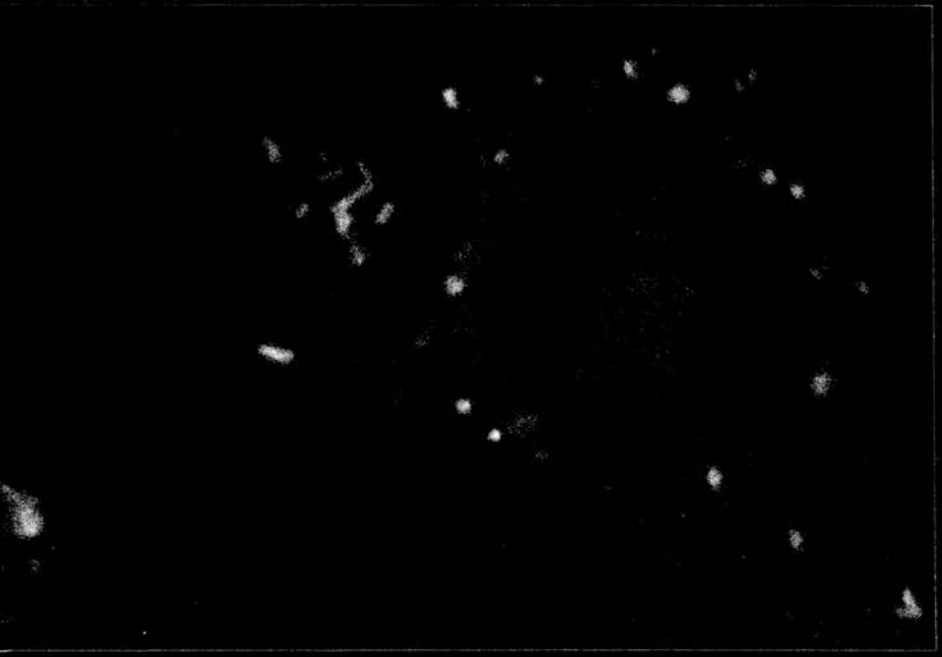


Fig. 14A

BK cells infected with C. sputorum ss mucosalis. One day post-inoculation, immunofluorescence staining shows a number of coccoid and vibrioid forms in the cytoplasm. Acetone fixation X 256.

Fig. 14B

After 3 days' incubation BK cells infected with C. sputorum ss mucosalis show areas of diffuse cytoplasmic fluorescence. Acetone fixation X 256.



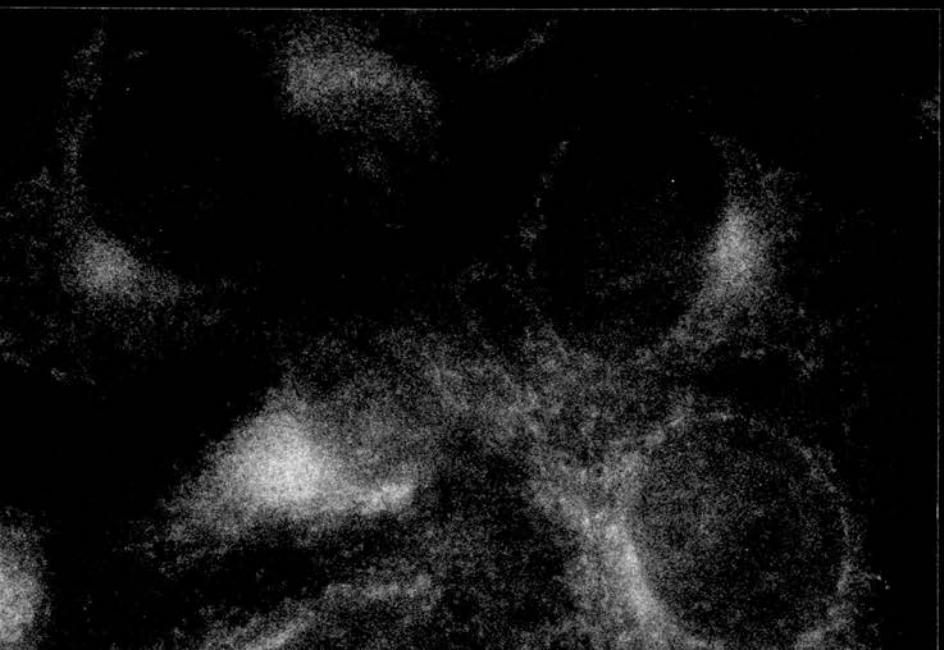
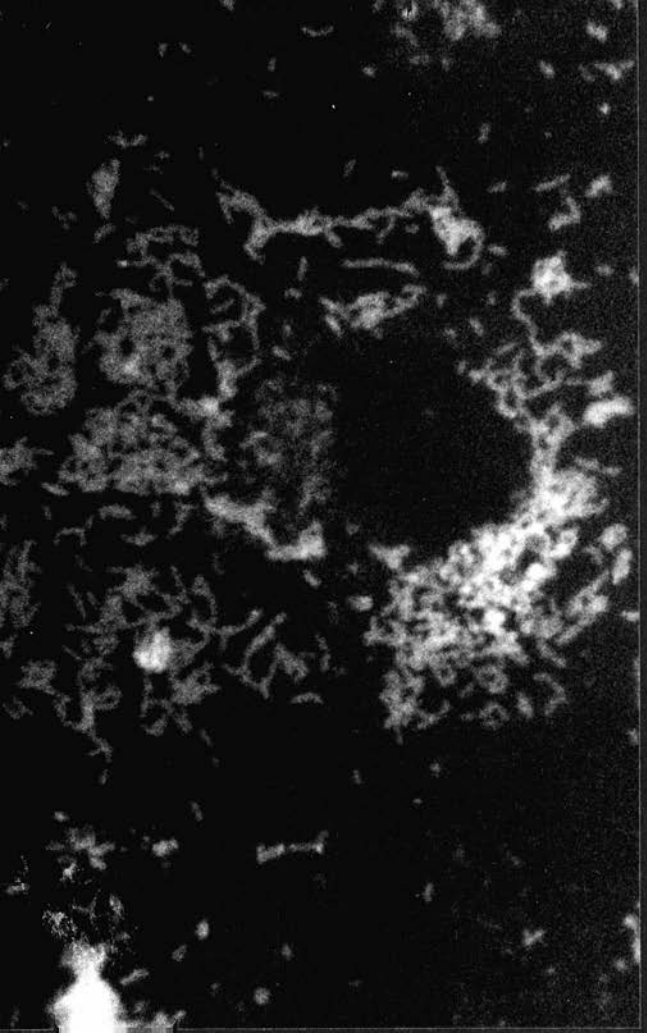


Fig. 15A

Immunofluorescence staining of seven-day-old infected BK cell cultures shows evidence of intracellular multiplication of large masses of brightly staining vibrioid forms in the cytoplasms of the affected cells. Acetone fixation X 256.

Fig. 15B

Accumulation of enormous numbers of vibrioid forms of C. sputorum ss mucosalis in the cytoplasm of 14-day-old infected BK cells. Note the absence of coccoid forms. Acetone fixation X 256.



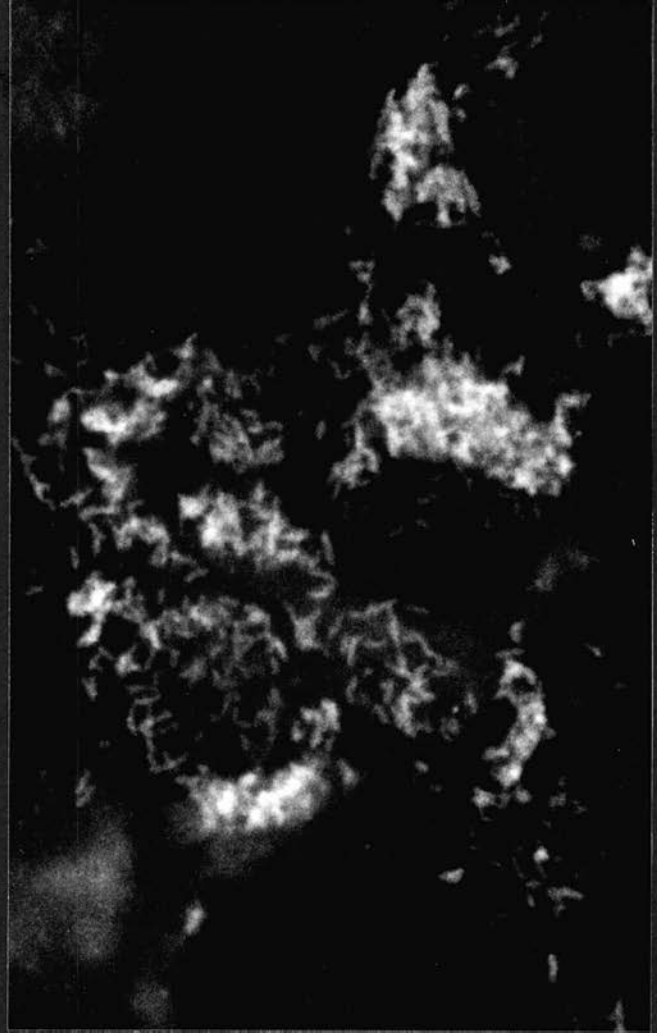


Fig. 16A

After 21 days the infected BK cells show very large numbers of C. sputorum ss mucosalis. In addition to the typical vibrioid forms there are also considerable numbers of coccoid forms. Acetone fixation X 256.

Fig. 16B

Persistence of mucosalis-specific antigens in infected BK cells. The monolayer was subcultured at the 3rd and 6th weeks and examined on the 55th day post-inoculation. Notice the presence of large numbers of tiny intracytoplasmic fluorescing particles; the nuclei are unaffected. Acetone fixation X 172.

Fig. 16C

Immunofluorescence staining of 7-day-old uninfected BK cell control. Acetone fixation X 256.

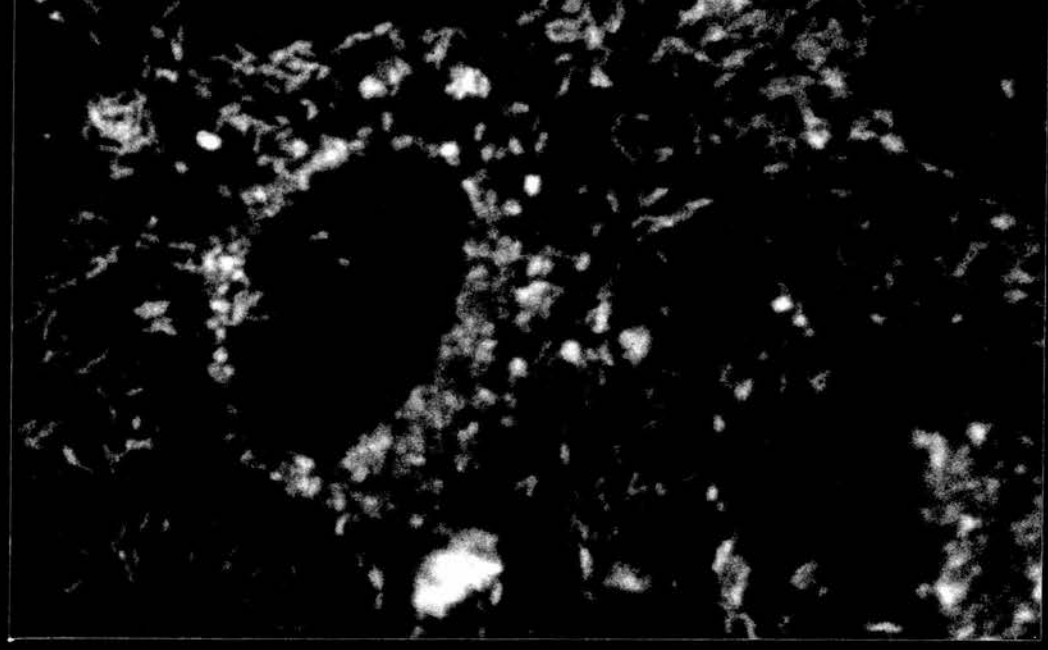
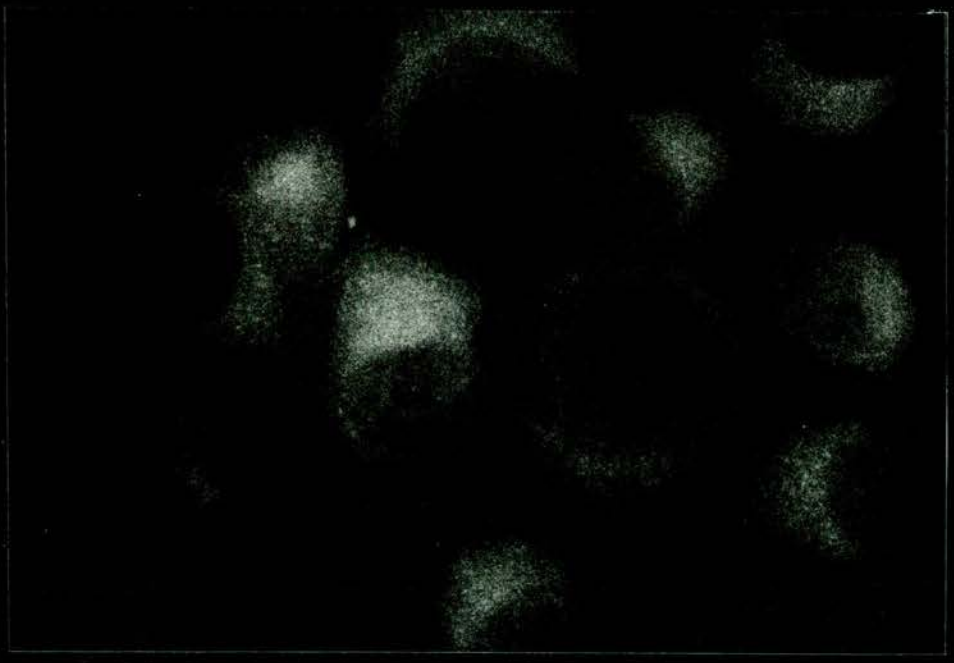


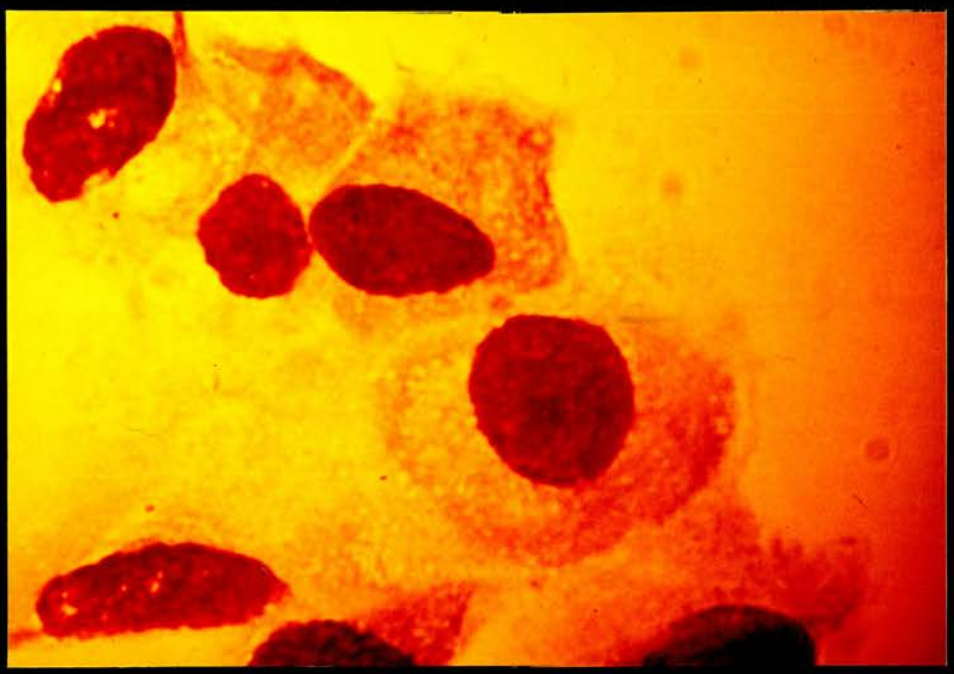
Fig. 17A

Similar preparation to that shown in Fig. 16A, but prefixed in formaldehyde and stained by the immunofluorescence method. The absence of fluorescing organisms, as seen in acetone-fixed preparations (Figs. 15 and 16), indicates that prefixation with formaldehyde prevents the penetration of mucosalis-antibodies. X 256.

Fig. 17B

A Giemsa-stained preparation of an infected monolayer similar to that shown in Fig. 16A. There are no extracellular attached bacteria and the Giemsa staining method is unable to demonstrate the presence of numerous intra-cytoplasmic mucosalis organisms as shown in Fig. 15 and 16. X 256.





cells was further substantiated by 7 to 21 day-old infected BK cultures which invariably failed to show intracytoplasmic fluorescence despite the fact that the same cells contained enormous numbers of fluorescing intracytoplasmic organisms when prefixed in acetone. Thus, prefixation with formaldehyde might provide a simple and reliable method for identifying intracellular multiplication of other pathogenic bacteria, particularly in cultures treated with antibiotics to remove any extracellular growth.

The results of immunofluorescence staining of PK_{pi} and BK cultures infected with mucosalis show that the intracellular fate of this organism differs in these two cell types. For example, in PK_{pi} cells intracellular mucosalis rapidly undergoes morphological changes and the 'coccoid' forms predominate during the course of infection. Although these 'coccoid' particles greatly increased in size, and in the intensity of their staining reaction, there was no evidence that these structures reverted to typical 'vibrioid' organisms. The nature of these 'coccoid' forms and their significance in the intracellular development of mucosalis is unknown and merits further detailed study.

The reappearance in BK cells of numerous long, filamentous bacterial forms which stained specifically with mucosalis antiserum, but were not seen in uninfected controls, is probably associated with intracellular growth. Further evidence of intracellular multiplication in BK cells was provided by the subsequent appearance

of enormous numbers of 'vibrioid' intracytoplasmic organisms which persisted for upto 42 days post-inoculation.

In summary, immunofluorescence staining provided a rapid and reliable method of assessing intracellular multiplication of mucosalis which should be rigorously applied to all infected cell culture systems before regarding them as being refractory to mucosalis infection.

III. DEMONSTRATION OF INTRACELLULAR C. SPUTORUM SS MUCOSALIS BY ELECTRON MICROSCOPY.

a. Introduction.

Several workers have studied the intracellular growth of pathogenic bacteria in infected cell cultures by electron microscopy, and the relevant literature has been reviewed in Chapter I. This technique is essential for a critical evaluation of the intracellular fate of pathogenic bacteria, particularly those organisms which are not cell-dependent for their growth. The information on the cytopathic changes induced in infected host cells by intracellular bacteria may lead to a better understaning of the pathogenic mechanisms involved. Although electron microscopy is not wholly satisfactory as a means of quantifying intracellular bacterial multiplication it has many advantages of over both light and immunofluorescence microscopy in that it can clearly distinguish the location of the bacteria and between apparently normal and degenerate organisms.

In the present investigation, light and immunofluorescence microscopy showed that C. sputorum ss

mucosalis rapidly underwent degenerative changes in certain types of cell cultures, while in others novel bacterial forms developed, which appeared to be responsible for the persistent 'parasitic growth' of this organism.

This intriguing situation required an investigation of the intracellular fate of this organism by electron microscopy in order to confirm and if possible explain the basis of these observation.

b. Design of experiment.

In this investigation, studies were restricted to the use of PK_{pi} and BK cell lines infected with mucosalis strain 253/72, since these two cell types produced distinct and characteristic responses to infection. The preparation of the bacterial inocula and the methods used to inoculate suspensions of trypsinized cells have already been described on page 95 and 96 respectively. These and a number of appropriate uninfected cell suspensions in medical flats were incubated at 37°C and allowed to form monolayers. Infected PK_{pi} and corresponding control cultures were processed for electron microscopy at daily intervals for 4 days, while similar preparations of BK cells were additionally examined on the 7th, 14th and 21st days post-inoculation. This selection of material was based on the earlier experimental evidence that mucosalis infection of PK_{pi} cells caused total destruction of the monolayers in 4 to 5 days whereas the infected BK cells remained attached to the glass and the monolayers persisted for several weeks (Chapter IV).

Infected and control BK cells were refed at weekly intervals with MEM incorporating 5% calf serum. In all these cases, specimens for electron microscopy were prepared as described in Chapter II.

Additionally, the morphological features of 'parasitic' mucosalis organisms present in 21-day-old cultures of BK cells were examined after negative-staining with methylamine tungstate. For this purpose a pooled sample of supernatant cell culture fluids was processed as described in Chapter II.

In one experiment, suspensions of trypsinized PK_{pi} cells were inoculated with mucosalis strain 253/72 and the infected cells grown in suspension culture for a period of 4 hr as described earlier in this chapter (p.184). At the end of this period, the infected cell suspension (50 ml) was sedimented by centrifugation at 1500 x g for 5 min. The cell deposit was gently resuspended in 50 ml of sterile normal saline, recentrifuged and the washed cells were then fixed in 2.5% glutaraldehyde cacodylate buffer prior to processing for electron microscopy.

c. Results.

i. Examination of PK_{pi} cells infected with mucosalis.

Suspension cultures of PK_{pi} cells examined 4 hr post-infection, showed enormous numbers of bacteria attached to the cell surfaces with active engulfment of mucosalis organisms. There appeared to be intimate contact between the bacteria and the host-cell membranes and in some places the membranes underlying the attached bacteria could be seen to be thickened and electron dense.

The engulfment of bacteria seems to be an active process in which the bacteria are partially trapped and encircled by the pseudopodia or surface processes. In most specimens organisms were present in phagosomes even at this early phase of infection and the membranes lining these vacuoles appeared to be intact. However, many bacteria were present in the cytoplasm apparently not surrounded by host-cell membranes (Figs. 18 and 19).

Examination of thin-sections of PK_{pi} cells during the first two days after infection showed a complete absence of bacteria attached to the cell surfaces. During this period, the majority of intracellular bacteria had lost their characteristic vibrioid morphology and appeared to be more 'coccoid' in shape and showed marked evidence of degenerative changes (Fig. 20). These abnormalities included a loss of the characteristic wavy outer coat of the organism and alteration to the normal uniform granularity of the cytoplasm. In general, the intracellular bacteria differed in their ultrastructure from organisms grown in culture and which have been described in detail in Chapter III. These degenerative changes in the bacteria were not associated at this stage with any demonstrable cytopathic or structural changes within the infected cells.

Ultrathin sections of infected PK_{pi} cells examined on the 3rd day post-inoculation contained relatively few recognisable bacterial forms but demonstrated the presence of large amounts of granular electron dense material. This material was largely aggregated and sometimes comprised

masses occupying a substantial proportion of the cell cytoplasm. Other smaller foci were often seen at the periphery of membrane-lined vacuoles (Figs. 21A-B, 22A), the centres of which were empty. The periphery of the granular material was often irregular and seemed to penetrate into the cell cytoplasm and in most cases no peripheral membrane structures could be seen although a narrow electron-lucid zone was invariably present around this material. At this stage the infected monolayer showed evidence of cell fusion and nuclear abnormalities including misshapen or fragmented nuclei. The mitochondria and other cell structures appeared to be unaffected.

Four-day-old infected PK_{pi} cells showed clear evidence of maximum accumulation of granular material within the cytoplasm (Fig. 21B). These aggregates continued to show an absence of any membranous envelope. This material seemed to be predominantly composed of electron dense, approximately hexagonal structures which were not dissimilar to those obtained from the outer cell surface structures of mucosalis (Figs. 4B, 22B).

Apart from the evidence of cell fusion and nucleorrhexis, the infected cells did not show any other evidence of structural changes. In all cases, appropriate uninfected controls remained normal and did not contain bacterial or other structures similar to those described above (Fig. 23).

ii. Examination of BK cells infected with mucosalis.

Ultrathin sections of one-day-old BK cells infected with mucosalis organisms showed the presence of intracellular bacteria and cell surfaces which were practically free of any demonstrable organisms. In the intracellular location bacteria occurred in groups mostly within the phagosomes, with occasional single organisms elsewhere in the cytoplasm. In the later situation the organisms were not surrounded by a host cell membrane.

During the 2nd and 4th days after infection there was considerable migration of bacterial bodies from the phagosomes into the cytoplasmic substance although this process was never complete and organisms could still be detected within phagosomes. In this cell line, in contrast to their fate in PK_{pi} cells, mucosalis did not appear to undergo marked degenerative changes and retained their vibrioid morphology. They appeared structurally normal although there was no clear evidence of intracellular bacterial multiplication or of cellular abnormalities associated with the bacterial infection (Fig. 24).

Ultrathin section of 7 day-old infected BK cells showed that the remaining bacteria lying within the phagosomes were undergoing extensive degeneration and that the contents of these sac-like structures mostly consisted of 'ghost cells' presumably representing degenerate forms of mucosalis. The residual structure of the 'ghost-cells' is not clear but their hollow ring-like appearance seemed to represent altered but intact cell walls of mucosalis

devoid of normal cytoplasmic material (Figs.25A-B, 26A). These structures differed completely from the aggregations of electron dense material observed in PK$_{pi}$ infected cells (Figs.21A-B, 22A-B).

Mucosalis organisms located in the cytoplasmic substance retained their usual morphology, although many degenerate bacterial cells were also present. The degenerate organisms were markedly swollen and showed irregular loss of the cell wall (Fig.26B). There also appeared to be an increase in the number of intracellular bacteria compared with 2 to 4 day-old preparations, although actively dividing bacterial cells were not recognised.

Cell preparations obtained from 14 to 21 day-old infected cultures showed a large number of intracellular organisms than observed hitherto, which suggested possible bacterial multiplication within these cells (Fig.27A). The organisms were predominently vibrioid and, in sections, appeared to be similar in morphology and ultrastructure to those obtained from bacterial cultures (Figs.28A-B).

Of further interest at this time (14-21 days) was the appearance of a large number of dense 'circular bodies' located at the periphery of the infected cell (Figs.29A-B-C-D). In some places there appeared to be a thinning-out of the host cell membrane suggesting that these bodies

were being released. These unusual structures were always circular in outline and some times showed an indistinct outer membrane (Fig.29B). The size of the larger bodies compared well with that of mucosalis but there was considerable variation in size and many were smaller.

In all these situations there was no evidence of any outstanding abnormalities in the structural components of the host cell cytoplasm although a number of infected cells showed what appeared to be an area of structureless cytoplasm at the periphery of the cell and this 'zone' was invariably free of any demonstrable cytoplasmic organelles (Fig.27B).

By comparison, uninfected BK cells retained their normal appearance and failed to show intracytoplasmic structures resembling either mucosalis organisms or the dense circular bodies described above (Fig.30).

Morphological features of 'parasitic' mucosalis organisms obtained from supernatant cell culture fluids.

The negatively-stained preparations of mucosalis organisms obtained from supernatant fluids of BK cells 4 days after infection showed predominantly filamentous forms with smooth cell surfaces. These organisms generally appeared thinner but otherwise closely resembled those bacteria obtained from 24 hr-old CBA culture (Chapter III). The majority of 'parasitic bacteria' possessed single polar flagella.

Fig. 18

Electron micrograph prepared from suspension cultures of infected PK_{pi} cells, 4 hr post-inoculation, showing the attachment, engulfment (arrow) and intracellular bacteria of mucosalis organisms. X24750

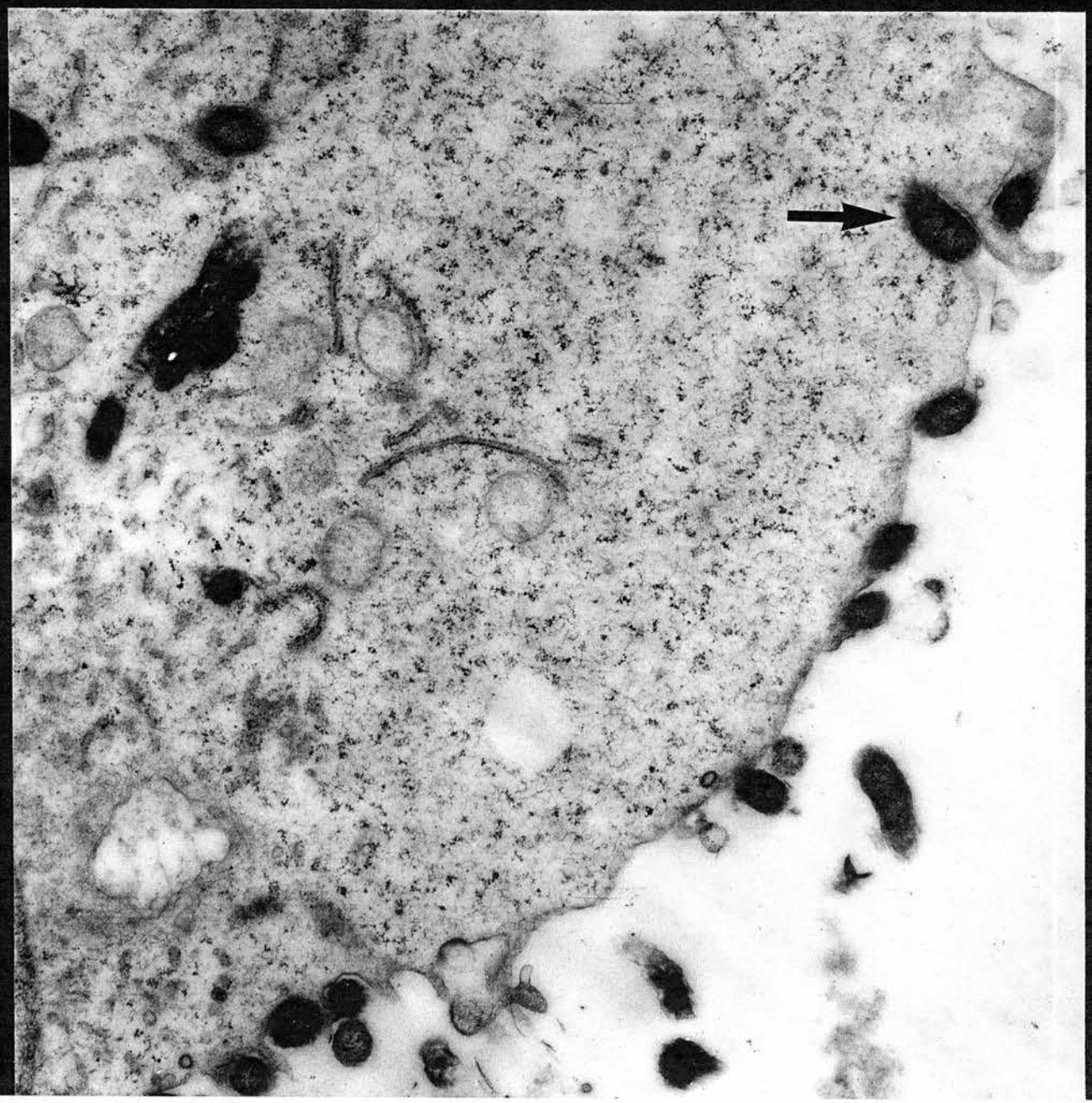


Fig. 19

Higher magnification of a similar preparation to that shown in Fig. 18. Note close contact between the organisms and the host-cell surface, and thickening of the plasmalemma at the point of engulfment (arrow). Also shown is an engulfed bacterial cell (B). X 60,000.

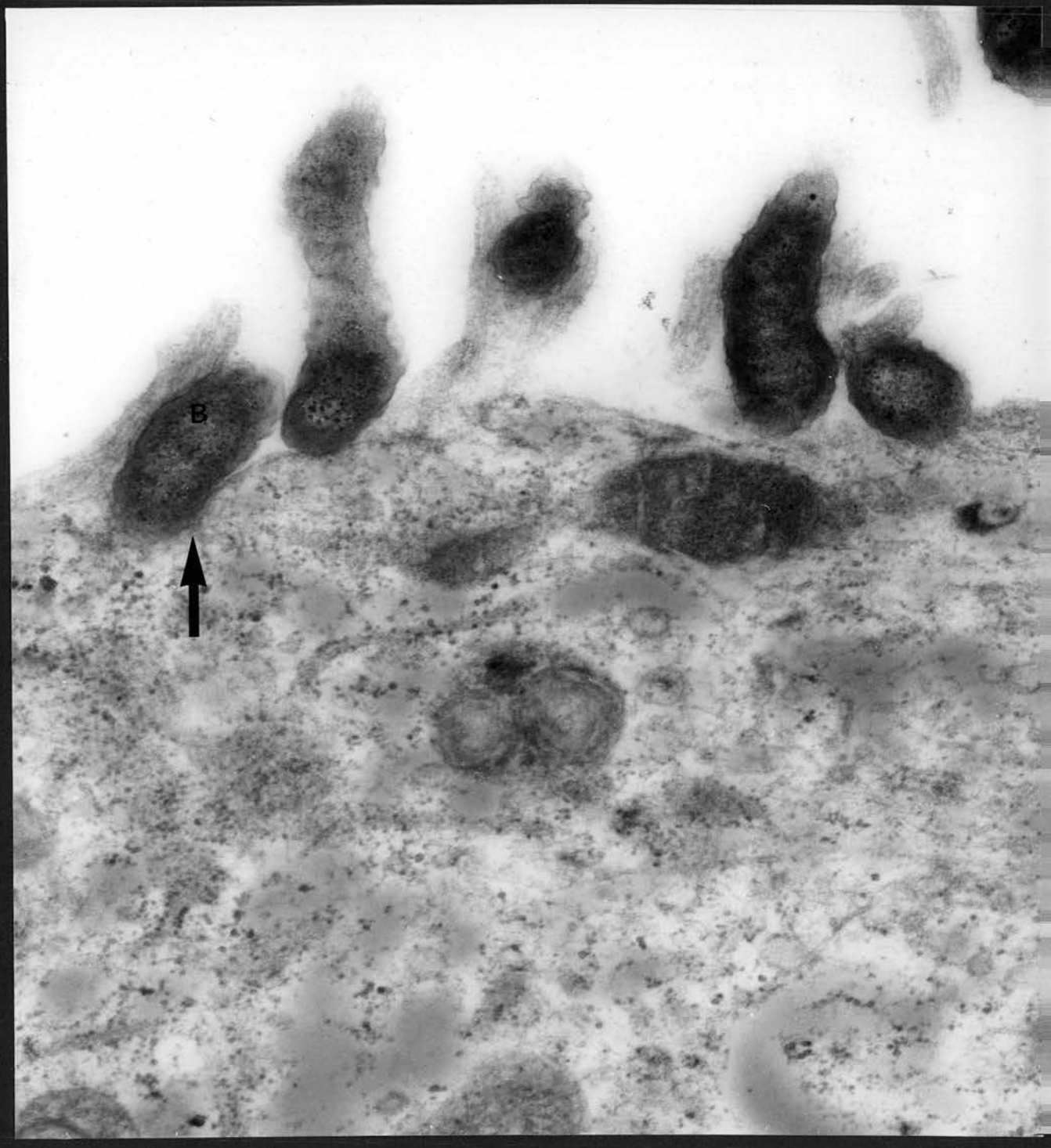

B

Fig. 20

Early disintegration (arrow) of C. sputorum ss mucosalis within the phagosome of an infected PK_{pi} cell, 24 hr post-inoculation. X 47,500.

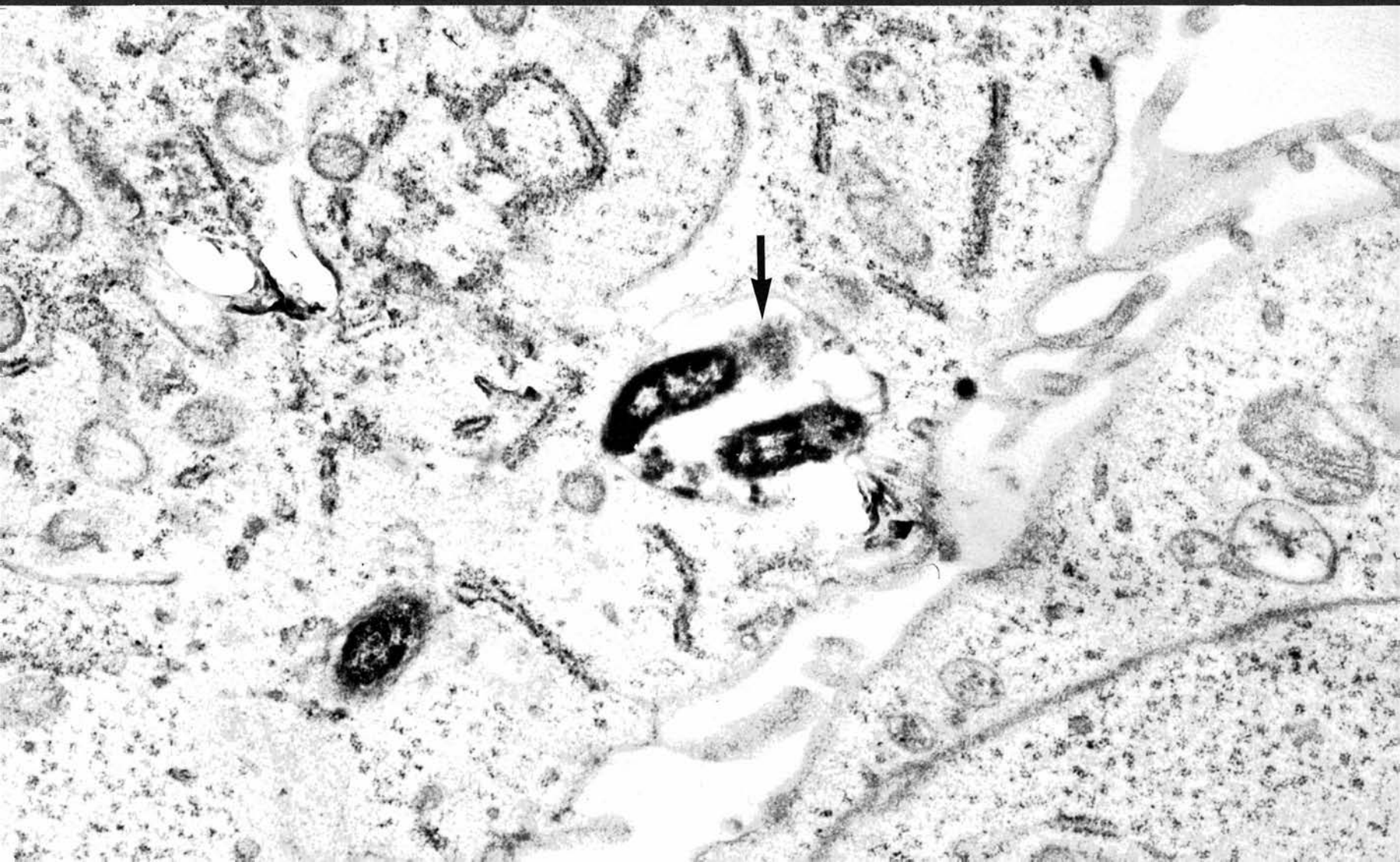
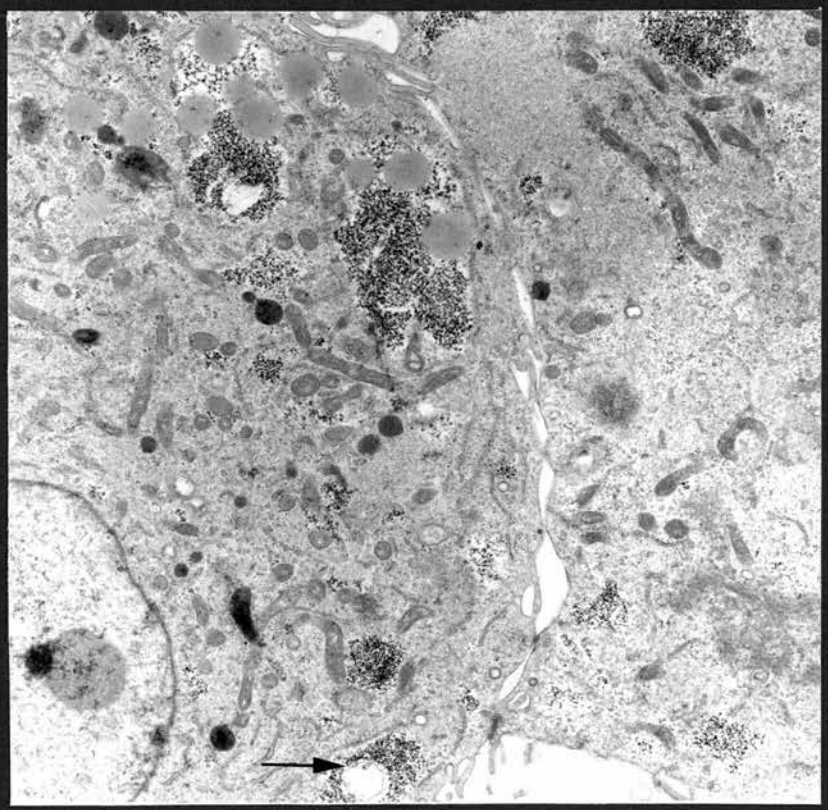


Fig. 21A

Electron micrograph of PK_{pi} cells showing several intracytoplasmic foci of granular material following phagocytosis and disintegration of C. sputorum ss mucosalis, two days after infection. Details of the phagosome (arrowed) are shown in Fig. 22A. X 9000.

Fig. 21B

A similar preparation to that shown in Fig. 21A, four days after infection with C. sputorum ss mucosalis. The cytoplasmic foci of granular material are much larger than those shown in Fig. 21A. The nucleus is not affected. X 9000.



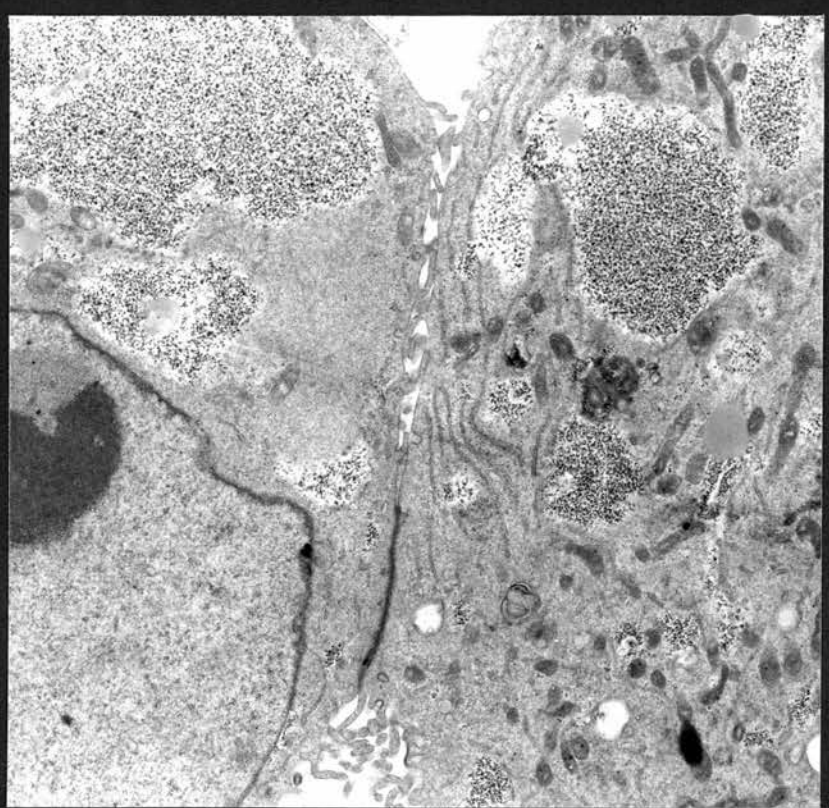
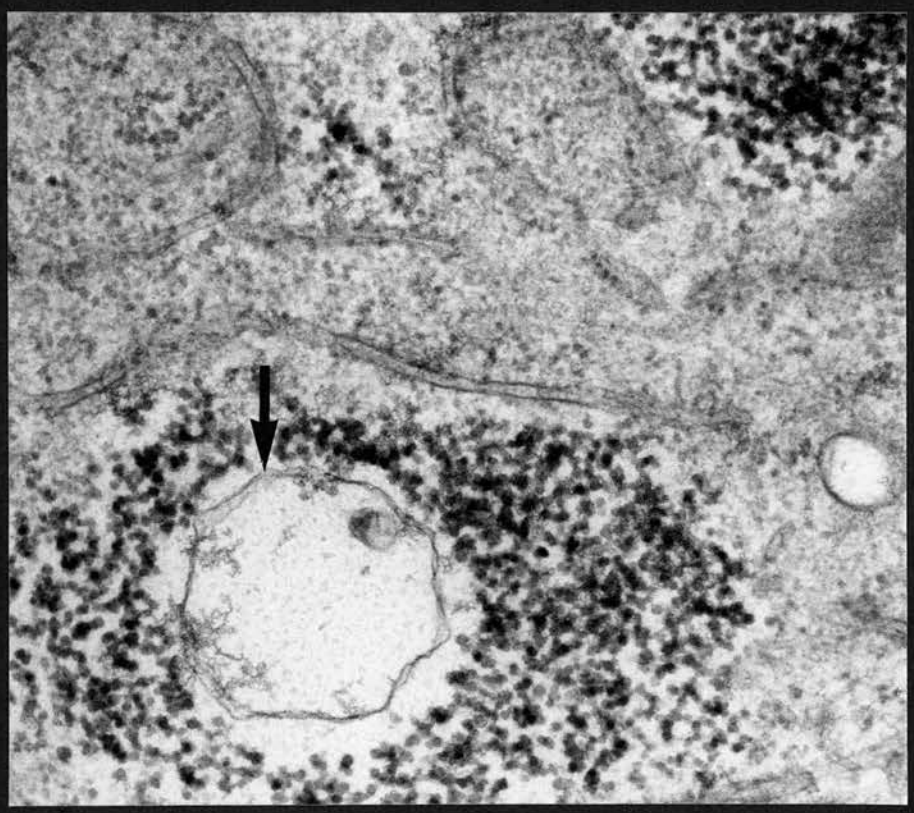


Fig. 22A

Higher magnification of the phagosome illustrated Fig. 21A. The 'granular material' in the lower phagosome surrounds a limiting membrane (arrow) of unknown origin. X 53250.

Fig. 22B

Higher magnification of 'granular material' present in PK_{pi} cells infected with C. sputorum ss mucosalis X 150000.



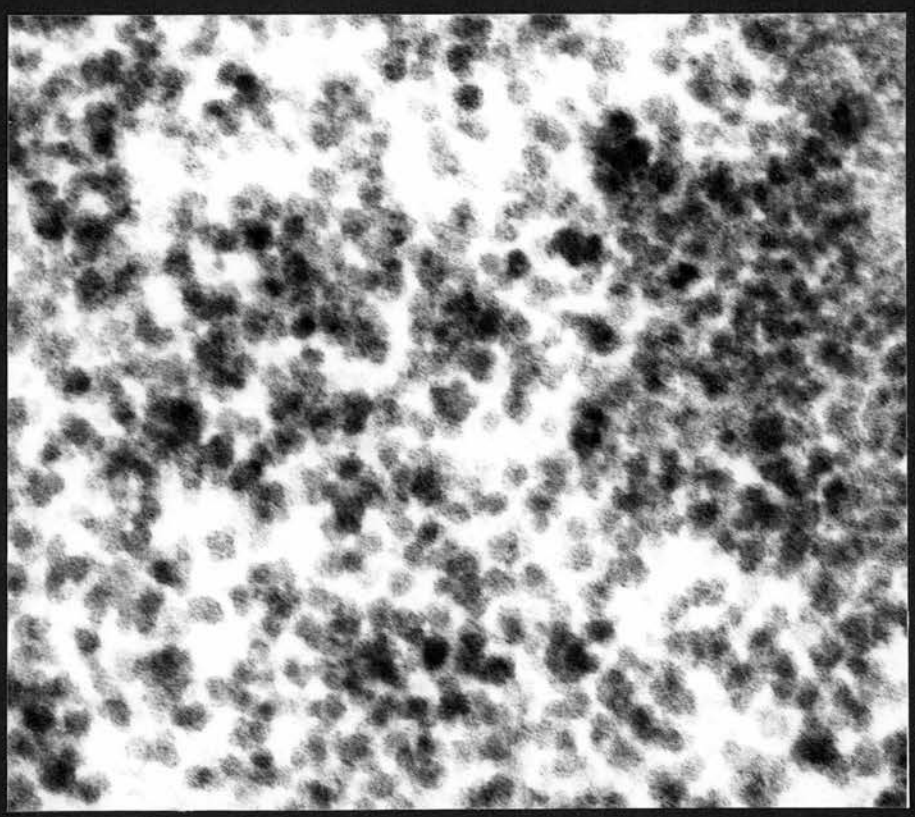


Fig. 23

Electron micrograph of 4-day-old uninfected PK_{pi} cell control. X 15000.

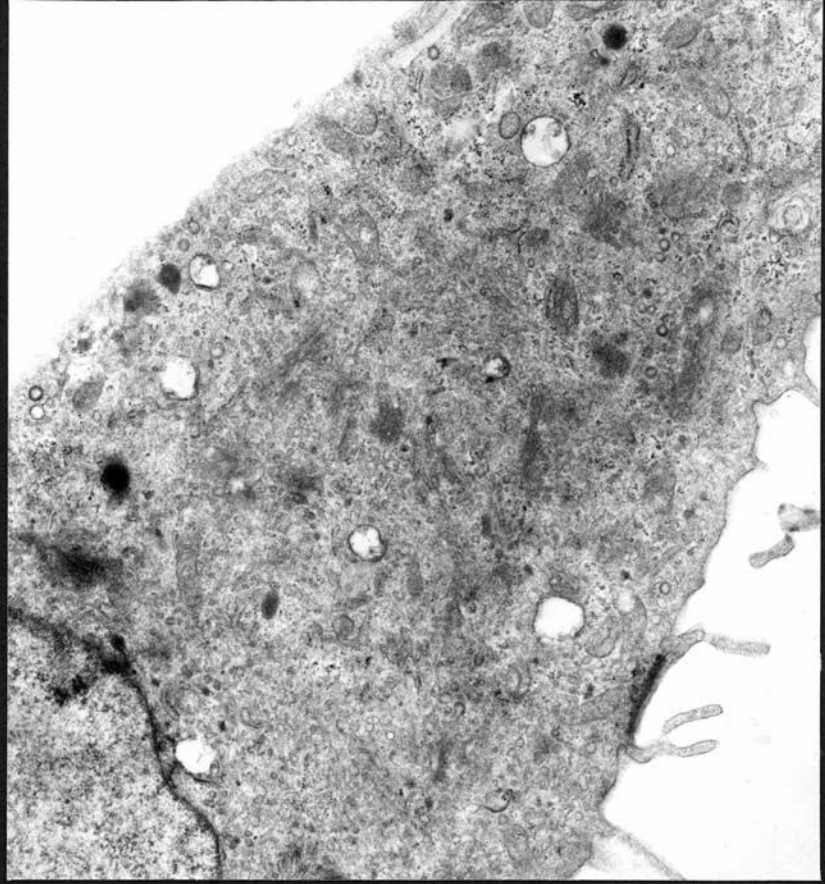
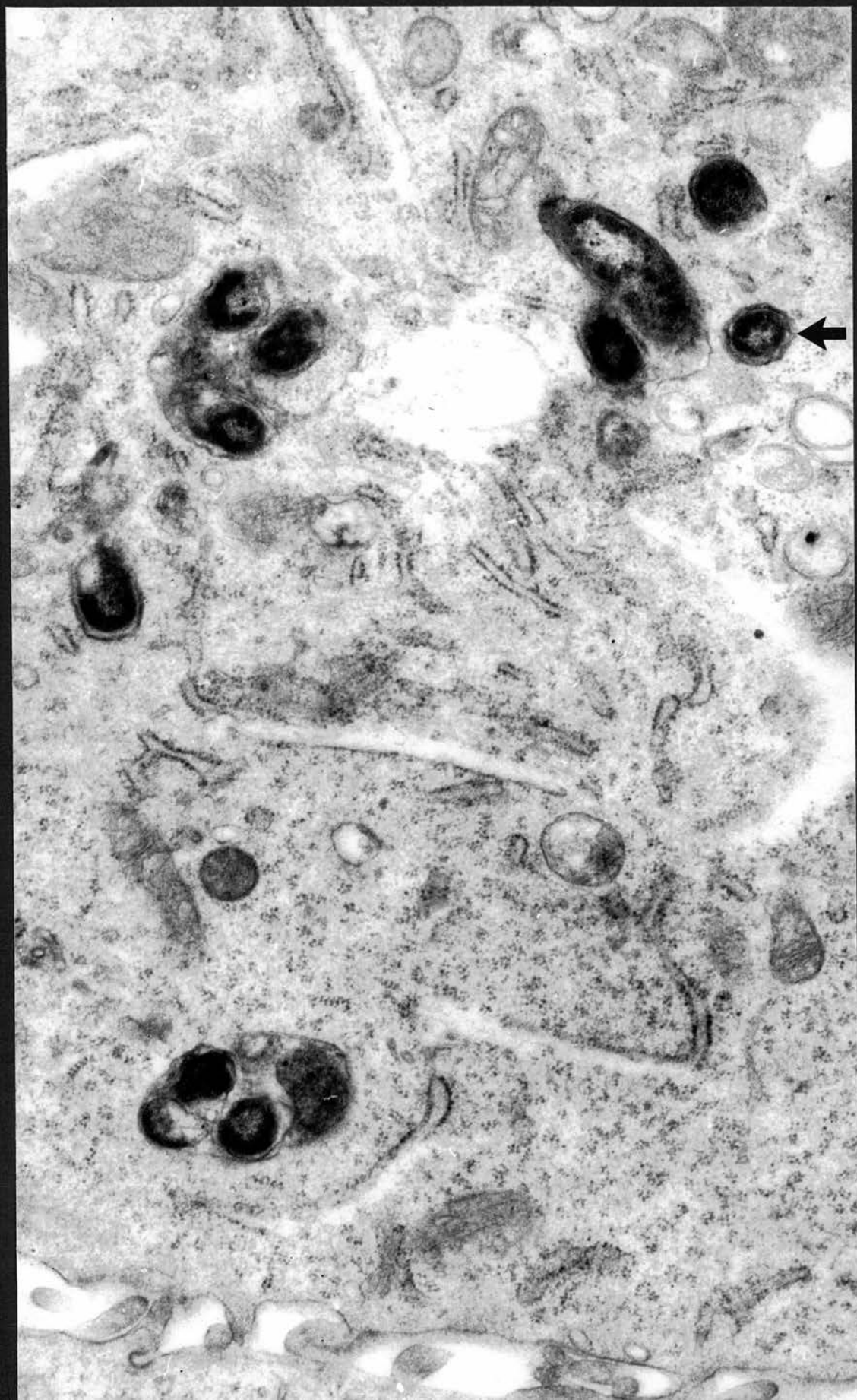


Fig. 24

Ultrathin section of infected BK cells, two-days-post-inoculation, showing the presence of a number of C. sputorum ss mucosalis organisms within the phagosomes. Some bacteria are lying free within the cytoplasm and are not surrounded by host membranes (arrow). X 47,500.



Figs. 25A and B

Details of ultrathin sections of infected BK cells showing loss of characteristic morphology of phagocytosed *mucosalis* organisms X 90000.

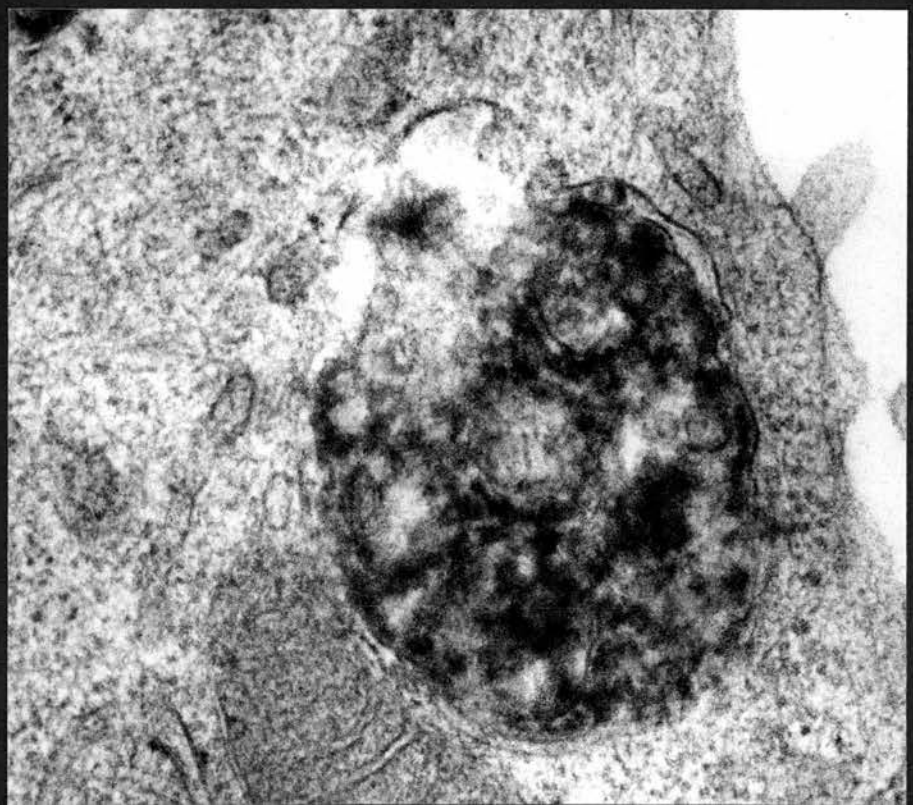
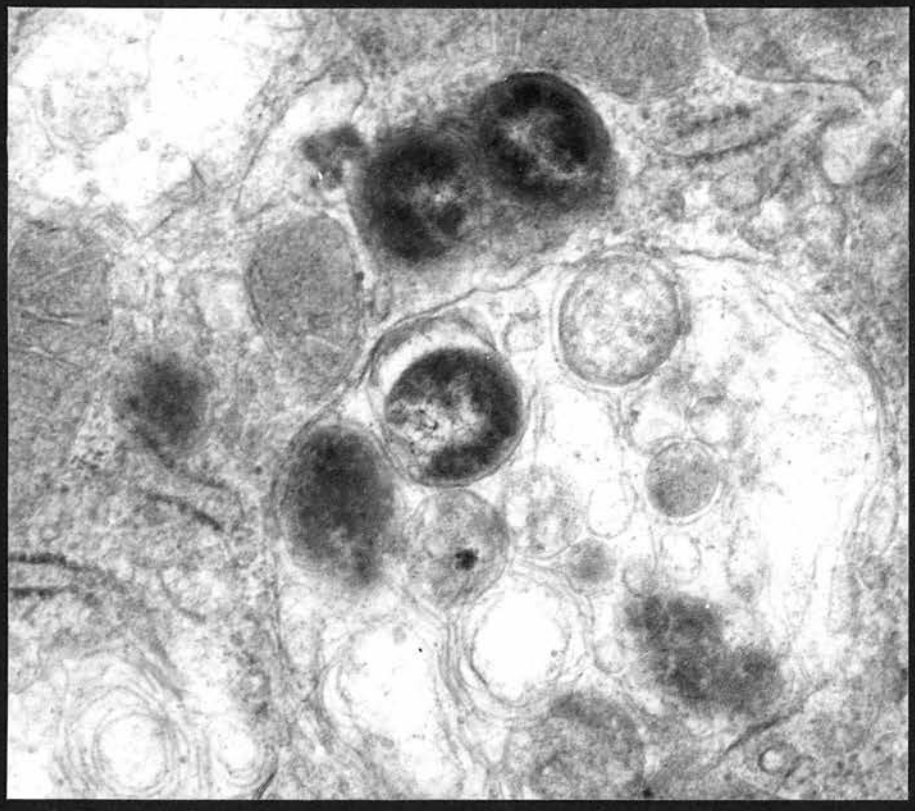


Fig. 26A

'Ghost cells' of C. sputorum ss mucosalis within a phagosome in a 7-day-old infected BK cell. Such 'ghost' structures could not be demonstrated ~~by organisms~~ lying free within the cytoplasm. X 69000.

Fig. 26B

Ultrathin section of 7-day-old BK cells infected with C. sputorum ss mucosalis showing a large, swollen degenerating bacterial cell. Note the irregular loss of cell wall structures (arrow).
X 150000.



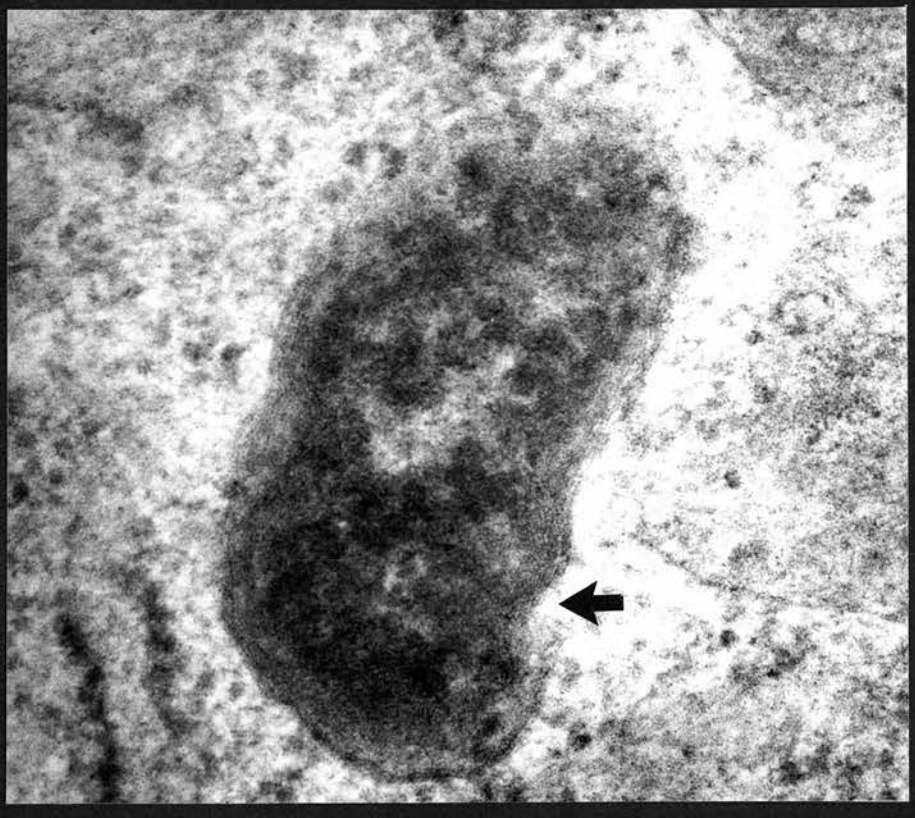
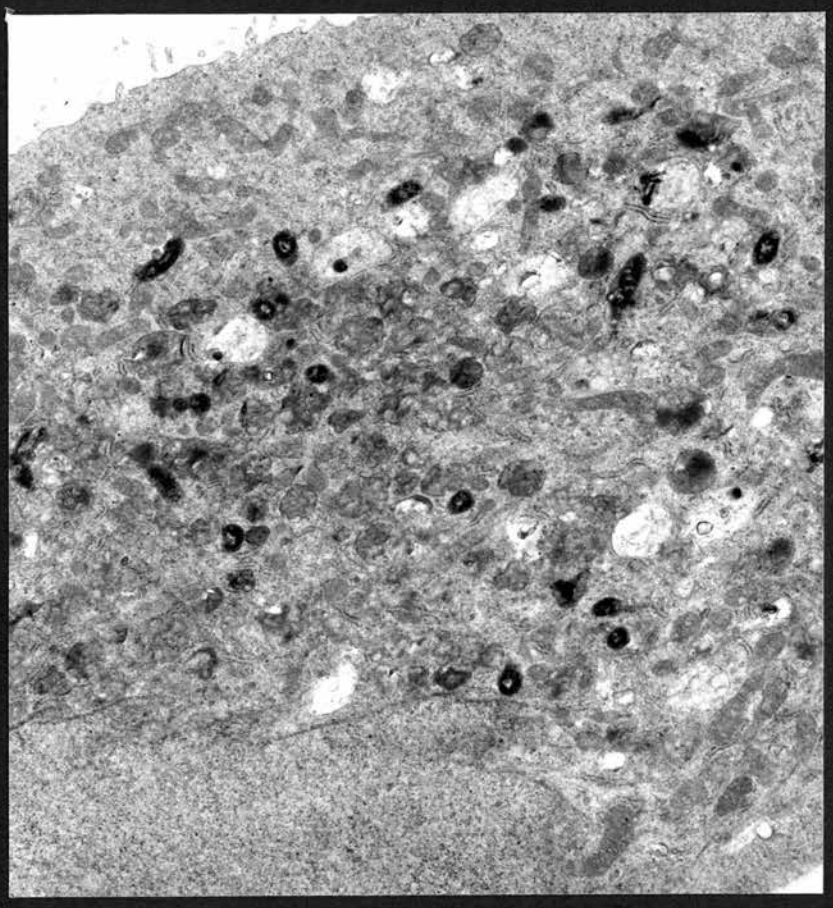


Fig. 27A

Ultrathin section of 14-day-old infected BK cell showing large numbers of C. sputorum ss mucosalis widely distributed throughout the cytoplasm. X 9000.

Fig. 27B

Twenty one-day-old infected BK cell showing a wide, featureless zone of cytoplasm at the periphery of the cell. Note the presence of two phagosomes (arrows) containing mucosalis organisms at different stages of degeneration. X 18750.



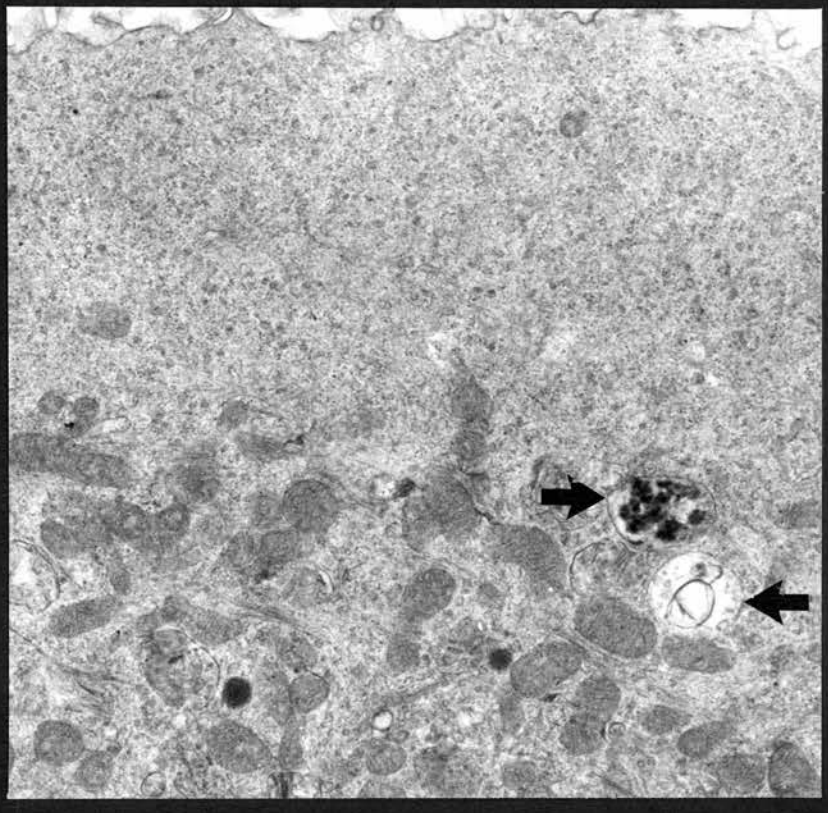


Fig. 28A

Electron micrograph of 21-day-old infected BK cell showing 'apparently normal' mucosalis organisms (arrow). Large numbers of these organisms are frequently found in the cytoplasm at this stage. X 24750.

Fig. 28B

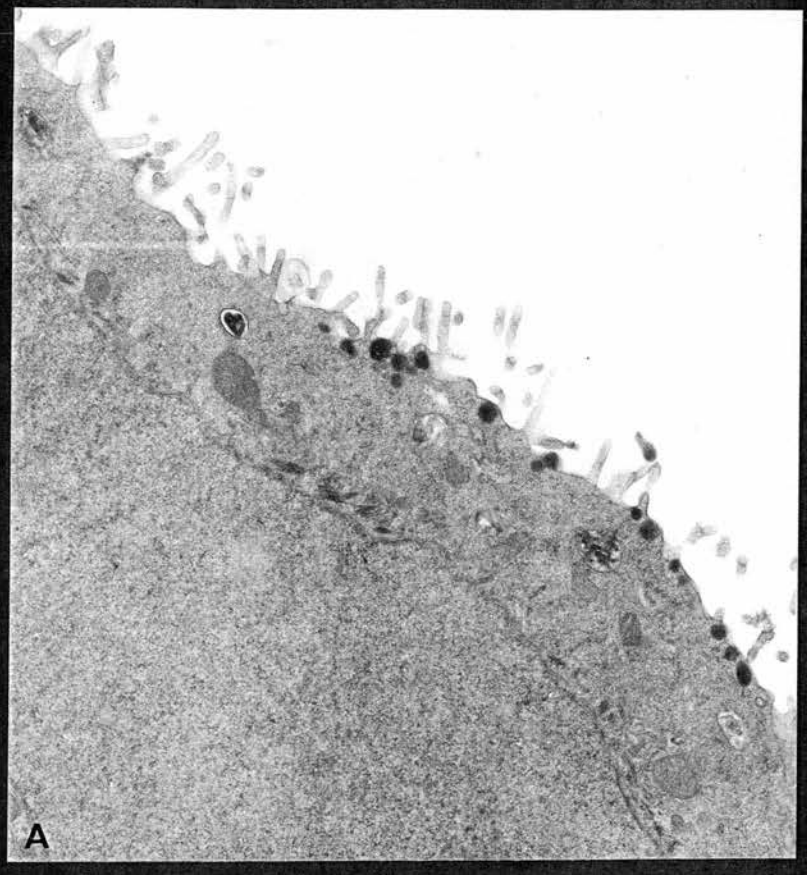
Higher magnification of two morphologically normal intracellular mucosalis organisms shown in Fig. 27A. The characteristically wavy cell wall and granular cytoplasm of the bacterial cells are clearly visible. X 90000.

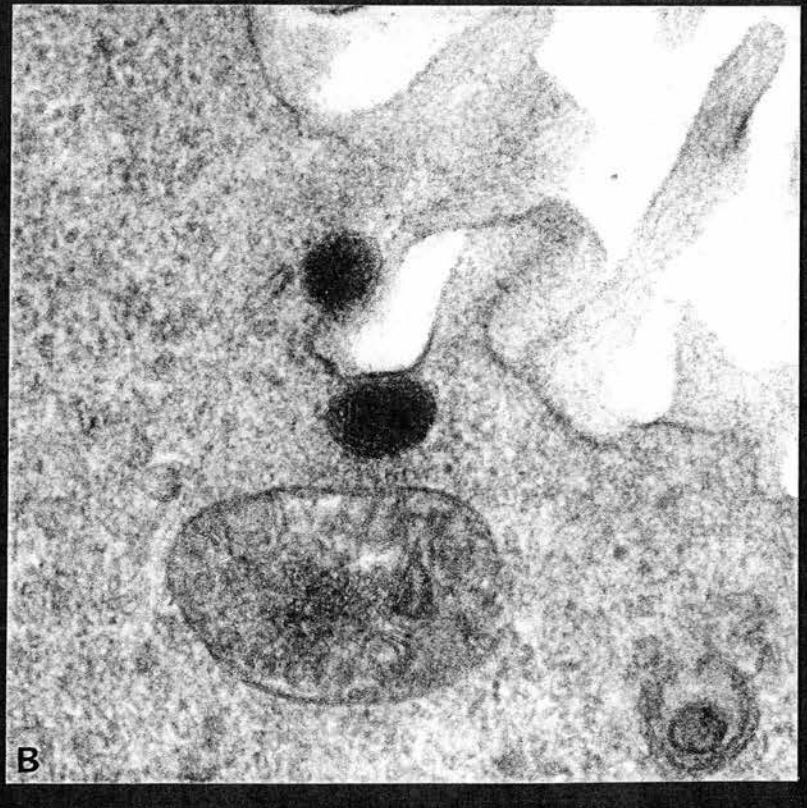
Figs. 29A - B - C - D

Electron micrographs of BK cells 14-21 days after infection with mucosalis showing various stages in the release of possible intracellular bacteria from infected cells.

A. Distribution of intracellular 'bacterial-bodies' along the surface of the cell. X 15000.

B. Detail of Fig. 28A. X 90000.

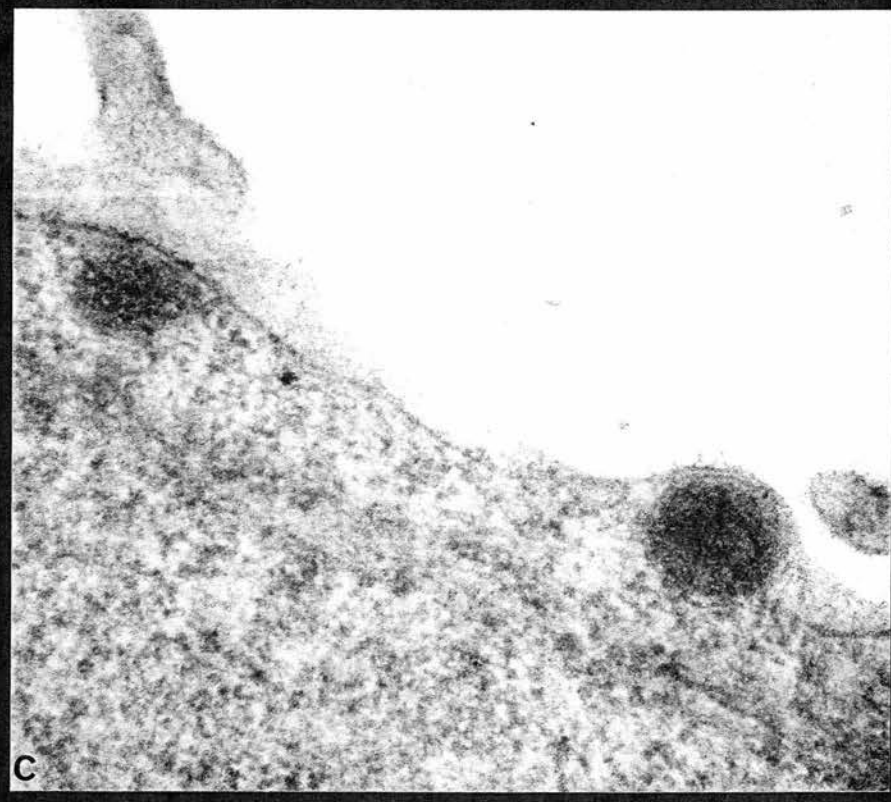

A


B

Figs. 29C - D

C. 'Bacterial-body' extruding through the cell surface. X 90000.

D. Cluster of peripheral 'bacterial-bodies' showing cell wall-like structures (arrows). X 150000.


C

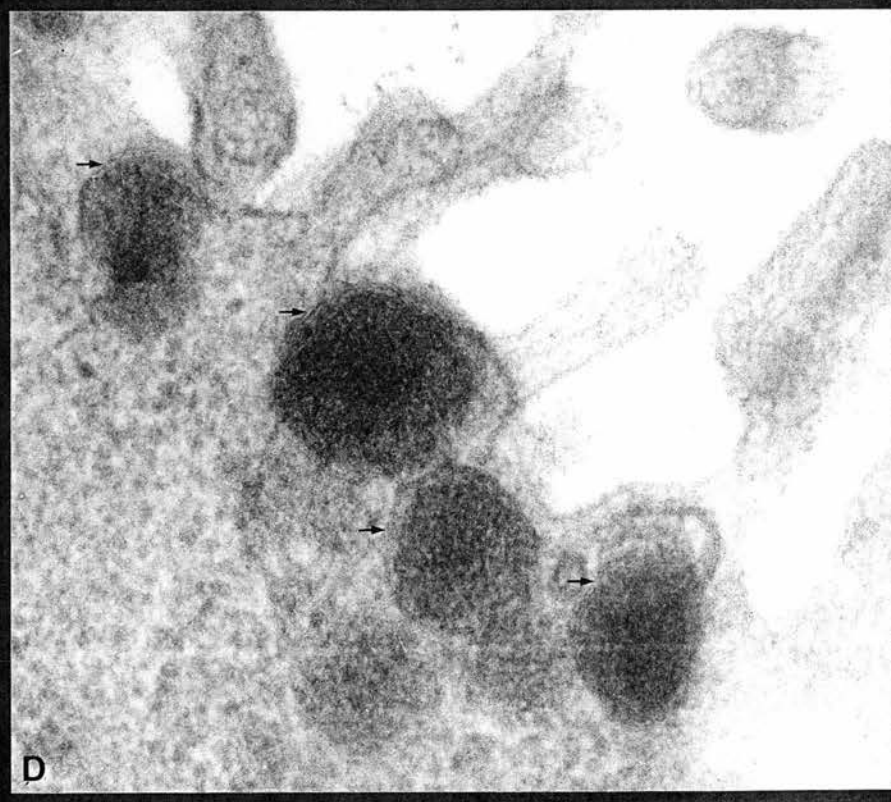

D

Fig. 30.

Uninfected 4-day-old BK cell control.

X 5325.

d. Comment.

This preliminary electron microscopic study of PK_{pi} and BK cells infected with mucosalis organisms has been invaluable in demonstrating the intitial interactions between this organism and cells, and its subsequent intracellular fate in these two types of cell cultures. The salient features are as follows:

i. Electron micrographs clearly showed that mucosalis attached firmly to the cell surfaces and that alteration of the host cell membranes occurred at the site of attachment. There was no evidence that flagella were involved in the adhesion process.

ii. Entry of attached mucosalis organisms into the cell was primarily associated with active engulfment by the host cell and many phagocytosed bacteria first appeared in the cytoplasmic phagosomes. The mechanisms involved in subsequent migration from the phagosomes to the cytoplasmic substance are not clear but there was no evidence of discontinuity in the membranes surrounding the phagosomes, which subsequently contained large numbers of 'ghost cells' of mucosalis.

iii. Bacterial attachment appeared to be a 'transitory' process and no demonstrable organisms were present on the cell surfaces after 24 hr.

iv. Intracellular bacteria disintegrate rapidly in infected PK_{pi} cells. This is followed by the accumulation of large amounts of granular material within the cell cytoplasm which stain specifically with FITC conjugated anti-mucosalis serum.

v. It is difficult to explain the source of 'parasitic' bacteria in PK_{pi} cells in view of the fact that intact bacteria are not observed in the cellcytoplasm 4 days after inoculation. Clearly the rapid and complete destruction of PK_{pi} cells makes it difficult, at present, to fully assess the results obtained in this cell system.

vi. In BK cell cultures there was clear evidence of intracellular destruction of mucosalis organisms, but cytoplasmic aggregates of granular material did not occur, as in PK_{pi} cells. Large numbers of 'ghost cells' were present in the phagosomes but the bacteria located in the cell cytoplasm were normal apart from minor changes in their cell walls.

vii. BK cells probably support intracellular growth of mucosalis since there seemed to be an increase in their numbers within the cell cytoplasm. Numerous electron-dense bodies were frequently seen at the periphery of the cells and the available evidence suggested that they were being released into the surrounding medium.

viii. The negatively-stained 'parasitic bacteria' were similar in morphology to those obtained from CBA plates (24 hr-old) except that they were predominantly long and filamentous, and possessed smooth cell coats. In some respects they resembled the organisms seen in old diphasic cultures.

DISCUSSION

It is emphasized that all specimens examined by light, fluorescence and electron microscopy were inoculated simultaneously with C. sputorum ss mucosalis to provide identical experimental conditions. Such a comprehensive approach was necessary in order to obtain as clear an overall picture as possible of the intracellular growth of mucosalis in different cell culture systems.

The attachment of mucosalis organisms to the surfaces of host cells was readily confirmed by all three methods and electron microscopy clearly showed the close contact achieved between the bacterial cell surface and the host cell membranes. The engulfment of mucosalis organisms was similar to that described in cell cultures with N. gonorrhoeae (Waitkins and Flynn, 1973; Brodeur et al., 1977) and Y. pseudotuberculosis (Bovallius and Nilsson, 1975). Electron microscopy was also particularly useful for demonstrating phagocytosis of large numbers of organisms as early as 4 hr post-inoculation and for confirming the absence of bacterial attachment to cell surfaces at 24 hr and thereafter.

The sequence of intracellular events occurring in PK_{pi} and BK cells varied greatly. For example, in PK_{pi} cells there was rapid disintegration of intracellular mucosalis organisms and the densely granular material that remained, seemed to accumulate in increasing amounts sometimes in association with the phagosomes. A similar picture was obtained in parallel coverslip preparations

examined by immunofluorescence staining wherein the intracellular organisms rapidly assumed coccoid forms and subsequently disintegrated to form large aggregates of small brightly fluorescing particles. The distribution of this granular material within the cell cytoplasm was very similar when viewed by immunofluorescence techniques or electron microscopy. However Giemsa-stained preparations failed to show the presence of these intracellular accumulations.

By comparison, similar studies on infected BK cells showed clear evidence of intracellular multiplication of mucosalis and the presence of 'ghost cells' was confirmed by electron microscopy. In the past, several workers have relied on immunofluorescence staining to assay the intracellular growth of pathogenic bacteria (Bovallius and Nilsson, 1975; Brunius, 1980) but the present study clearly underlines a major limitation of this method since it fails to distinguish between viable and dead bacteria and may result in erroneous interpretation based on such observations alone. The same criticism can also be applied to cell culture studies involving light microscopy. However, it is possible by electron microscopy to distinguish between apparently normal looking and altered or degenerate intracellular bacteria.

At this stage no particular explanation could be given for the different events associated with the intracellular fate of mucosalis in PK_{pi} and BK cells, or the mechanisms

involved in the production of parasitic growth in these different types of cell cultures.

It is also interesting to note that mucosalis failed to induce ultrastructural changes in the cytoplasm of cultured cells and in this respect it behaves in a similar manner in the intestinal epithelium of naturally occurring cases of PIA. Gross cellular changes are produced in cell cultures and these will be described fully in the next chapter.

CHAPTER VIII

CHAPTER VIII

CYTOPATHIC CHANGES PRODUCED BY C. SPUTORUM SS MUCOSALIS IN CELL CULTURES.

GENERAL INTRODUCTION.

In recent years cell culture systems have been used increasingly to explore the pathogenic mechanisms of microorganisms. In Virology, the sequence of events associated with viral replication including assembly and release of progeny virions, and their cytopathic effects on cell cultures, are well documented. By comparison, relatively little information is available about the growth and development of bacteria in tissue cultures, and the response of host cells to bacterial infections is poorly understood. It is evident from the available literature on this subject that most species of pathogenic bacteria, particularly Gram-negative organisms such as Salmonella and Shigella, cause rapid degenerative changes in cell cultures with the result that other types of cellular abnormalities are only rarely seen.

Recently, through the development and introduction of improved techniques including the selective use of antibiotics to restrict extracellular growth, cytopathic changes associated with bacterialinfections are being increasingly recognised. A good example of this is the description of cellular abnormalities in cultured monkey kidney cells (Vero and $LLCMK_2$ cells) infected with B. canis which was recently reported by Egwu and Eveland (1979).

It became apparent during the early stages of this work that C. sputorum ss mucosalis i) can assume a 'parasitic' role and multiply in association with certain types of cultured cells, ii) can attach to a variety of permissive cells and, iii) on being phagocytosed by these cells, can regularly induce a number of unusual cytopathic effects. Because of the paucity of information available on the cytopathic effects induced in cell cultures by bacteria, it was felt that some of the cellular abnormalities observed in mucosalis infected cell cultures merited further investigation.

In this chapter the term 'altered cells' has been introduced to describe mucosalis infected cells showing cytopathic changes characterized by thin, featureless and abnormally enlarged cytoplasms. But despite these changes, the 'altered cells' survive and remain firmly attached to the glass surfaces of the cultured vessels.

I.A. CYTOPATHIC CHANGES IN PRIMARY AND CONTINUOUS CELL CULTURES INFECTED WITH C. SPUTORUM SS MUCOSALIS.

b. Design of experiment.

It is emphasized that the design of these experiments to demonstrate the changes induced by mucosalis organisms in different types of cell was basically similar to some of the experiments reported in Chapters V, VI and VII. These earlier experiments were used to obtain preliminary data on the cellular changes observed in this study and any additional information was obtained by modifying these

basic techniques as required.

i. Cell cultures examined.

In all, 12 different types of cell cultures were studied for their responses to infection with mucosalis organisms. These included, lines of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK, DK, Vero, $LLCMK_2$, HeLa cells, and primary cultures of pig kidney cells (PPK) and chicken embryo fibroblasts (CEF).

ii. Bacterial strains examined.

Four strains of C. sputorum ss mucosalis (253/72; 982/76; 512/77; 1075/78) were compared for their ability to induce cytopathic changes in cell cultures. Also examined were C. coli strain 124/73 A4 and the biochemically and serologically distinct catalase-negative Campylobacter strain 20/74.

iii. Cell culture infection procedures.

Infection of trypsinized cell suspensions and preformed monolayers with one or other the above named bacterial strains was carried out by the methods described in Chapter IV, p. 96. Infected and uninfected coverslip preparations were incubated at 37°C and examined at daily intervals for 2 weeks or, in some cases, for as long as the cell sheet remained attached to the glass. Coverslip cultures were stained with Giemsa's stain and examined by light microscopy.

In selected experiments, several similarly infected medical flats and coverslip preparations were refed at four-day intervals with fresh MEM and reincubated at 37°C. Coverslip preparations obtained after the first refeeding,

and daily thereafter, were compared with corresponding preparations which had not been refed. Cytopathic changes and the attachment of the cell sheets to the glass were regularly assessed by light microscopy.

iv. Inoculation of cell cultures with bacteria-free filtrates obtained from diphasic growth of mucosalis strain 253/72.

In selected experiments, the fluid phase of CBA-MEM diphasic medium containing a 24 or 48 hr old growth of mucosalis strain 253/72 was harvested and centrifuged at 6000 x g for 15 min to deposit the bacteria. The supernatant medium was then carefully collected and passed through Millipore membrane filters of 0.45 μm or 0.22 μm A.P.D. The bacteria-free filtrates were supplemented with 5% heat inactivated calf serum before overlaying 1 ml of each filtrate separately on to one-day-old coverslip preparations of PK, PK_{pi}, BK and BK_{pi} cells. These and appropriate uninoculated control cultures were incubated at 37°C and examined for the presence of cellular changes daily for 7 days, and then on the 14th and 21st day post-inoculation.

Prior to the inoculation of cell cultures, the CBA-MEM diphasic filtrates were checked for the presence of viable bacteria by plating 0.2 ml on each of two dry CBA plates which were then incubated in a hydrogen microaerophilic atmosphere at 37°C.

c. Results.

i. Cytopathic changes observed in primary pig kidney (PPK), PK and PK_{pi} cells.

It will be recalled that PPK, PK and PK_{pi} cells attached and phagocytosed mucosalis strains mostly during the first 12 hr post-inoculation.

In the present experiment, examination of Giemsa-stained coverslip preparations indicated that mucosalis infection did not interfere with the growth and attachment of infected trypsinized cells to the glass nor with subsequent monolayer formation. Neither the trypsinized suspended cell cultures nor preformed monolayers infected with mucosalis presented any unusual morphological changes during the first 24 hr after infection. However, towards the end of 48 hr, PK cells started to show marked cytoplasmic vacuolation and appeared to be rounding up. By contrast, PPK and PK_{pi} cells appeared normal although there was early evidence of cell fusion. All infected cell types remained attached to the glass at this stage.

The third day after inoculation was characterized by extensive cytopathic changes in the infected PK cells. This was associated with destruction and detachment of a large number of infected cells from the glass. The few cells that remained were pyknotic but there was no evidence of nuclear fragmentation. Unlike PK cells, there was no evidence of cytoplasmic vacuolation, pyknosis or detachment of PPK or PK_{pi} cells from the glass.

On the 4th day post-inoculation, there was complete destruction of the PK monolayers and the supernatant fluids contained large clumps of rounded-up cells. During this period, PK_{pi} cells showed extensive syncytial formation involving about 70 to 80% of the cells of the monolayer. These artifically induced polykaryons contained up to 16 to 20 nuclei, some of which were swollen or enlarged (Fig.31B). Other nuclear changes in polykaryons, as well as in solitary cells, included misshapen nuclei and nucleorrhexis, but 'foamy' degeneration was not apparent. Most of the remaining unfused cells showed marked 'swelling' of the cytoplasm and nucleus.

Infected PPK cells also formed multinucleate syncytia but to a much lesser extent than PK_{pi} cells and this did not exceed 60% of the cell population. The nuclei within the PPK polykaryons did not exceed 10 to 15 in number, were less misshapen but showed extensive nucleorrhexis.

The unfused PPK cells showed very little cytoplasmic vacuolation or cytoplasmic and nuclear enlargement. Pyknosis was not a characteristic feature of infected PPK and PK_{pi} cells although in most cultures a small number of cells became detached from the glass.

Marked degenerative changes appeared in PPK and PK_{pi} cells on the 5th day after inoculation. Nuclear fragmentation was extensive and cytoplasmic enlargement continued until, in most cases, very little of the monolayer

remained. By day 6 post-inoculation complete destruction had occurred.

Examination of coverslip cultures prepared from trypsinized PPK, PK and PK_{pi} cells, inoculated with either C. coli or catalase-negative Campylobacter strain 20/74, showed that the infected cells did not attach readily to the glass. After 24 hr incubation the monolayers appeared to be thin and patchy. Compared with those of corresponding cultures infected with mucosalis strains, or of uninfected controls, the cytopathic effects of C. coli and Campylobacter strain 20/74 were spectacular, and both produced rapid and total destruction of all three types of infected monolayers within 48 hr post-inoculation. The uninfected cultures appeared normal and none showed cellular abnormalities during the period of examination (Fig. 31A). Because PK_{pi} cells are chronically infected with a defective strain of Newcastle disease virus the monolayers used in this experiment consistently showed small foci of fused cells containing not more than 4 to 6 nuclei. For this reason the cytopathic effects of mucosalis strain 253/72, C. coli and Campylobacter strain 20/74 on PK_{pi} cells was also examined in cultures grown in the presence of rabbit anti-NDV serum and the results are discussed later in this chapter.

ii. Cytopathic changes observed in BK, BK_{pi}, OK_{pi}, BHK and HeLa cells.

Following infection with C. sputorum ss mucosalis, suspensions of these cells continued to grow normally and produced confluent monolayers within 72 hr. However, compared with the uninoculated controls, monolayers infected with mucosalis consisted of sparser cell sheets 4 days post-inoculation. At this stage most of the infected cells showed swollen cytoplasms and the effect appeared to be progressive, resulting in marked cytoplasmic and nuclear enlargement. These 'ballooned' cells accounted for approximately 70% of the monolayers during the 5th to 8th day, with the remaining cells appearing normal (Fig. 32).

Examination of monolayers after the 8th day showed cells with enormously increased cytoplasms and enlarged nuclei. This stage of abnormal cellular development was characterized by a 'thinning-out' of the cell cytoplasm which became less granular, featureless and, in general, did not show cytoplasmic vacuolation. Morphologically, these cells remained distinct from some 30% of the cells in the infected monolayer which persisted as apparently normal cells similar to the uninfected control cells. Those cells showing cytoplasmic 'ballooning' as a result of mucosalis infection were designated as 'altered cells' (Fig. 33B).

Coverslip preparations of BK, BK_{pi} and, to a lesser extent, BK, BHK and HeLa cells, obtained 6 days after inoculation, showed evidence of cell fusion in about a third of the monolayer. These fused cells contained upto 10 nuclei and the cytoplasm surrounding each polykaryon/nuclear mass appeared to gradually increase in size with continued incubation until, by 10 to 12 days, the cultures contained massive areas of thin, featureless cytoplasmic sheets. Apart from inducing polykaryons, the distended cytoplasm of individual 'altered cells' tended to fuse together to form enormous featureless cell sheets with sparsely distributed nuclei (Figs. 32, 33B).

The 'altered cells' in BK_{pi} and OK_{pi} cultures, whether produced by cell fusion or otherwise, usually contained a larger number of cytoplasmic vacuoles than did the control cultures. Degenerating 'altered' cells often showed a 'foamy' type of cellular change in the cytoplasm and, occasionally, in the aggregated nuclei (Fig.34). The presence of extensive vacuolation and 'foamy' degeneration was not a feature of BK, BHK and HeLa cells.

Nuclear changes in infected BK, BK_{pi}, OK_{pi}, BHK and HeLa cells were recognisable around the 4th day post-inoculation when the nuclei appeared swollen or enlarged. This change seemed to correspond with the extent of cytoplasmic enlargement and appeared to be progressive. Examination of monolayers around the 8th day post-inoculation showed marked variation in the size of the nuclei and some could be described as

'giant nuclei'. There was no change in the number of mitotic figures compared with control cultures. Misshapen nuclei tended to occur only during the later stages of infection (8 to 10 days) but a 'foamy' type of vacuolation was evident in some of the degenerating 'altered' cells, particularly in BK_{pi} and OK_{pi} cultures (Figs. 35A-B).

The initial stages in the production of 'altered cells' appeared to be similar in all five types of cell cultures although infected BHK cells were unusual in that a large number of 'altered' cells'tended to remain separate from each other and did not fuse to form the massive plaques observed in other types of cell cultures. It was also of interest that a large number of small, circular, cytoplasmic vesicles were attached to the surfaces of 'altered BHK cells'. Although it is reasonable to suppose that these extracellular cytoplasmic fragments might have arisen from the degenerating cell, their origin and function were not ascertained.

In all these experiments, uninfected control cells remained firmly attached to the glass and showed no evidence of cellular abnormalities (Fig. 33A). It should also be emphasized that the NDV-infected BK_{pi}, and OK_{pi} cultures contained small foci of fused cells, with 4 to 6 nuclei, which together with a limited degree of cytoplasmic vacuolation are characteristic features of these cell lines. C. coli and Campylobacter strain 20/74 produced destructive changes in about 3 to 4 days time, in both cell lines.

iii. Cytopathic changes observed in DK cells.

Dog kidney (DK) cells, unlike other types of permissive cells, attached and phagocytosed only moderate numbers of mucosalis organisms (Chapter VI, VII). Infected cells appeared normal during the first 3 to 4 days of incubation but, later, nearly 50% of the cell population showed slightly enlarged cytoplasms. Nuclear changes were not generally recognisable. This process of cytoplasmic enlargement did not appear to progress as far as in other cell types and lasted for up to 8 to 10 days post-inoculation.

There was no evidence of 'altered cells' or the presence, intracellularly, of 'novel' filamentous bacterial forms. Infected cells remained attached to the glass and the monolayers were confluent and compared well with the uninfected controls during the period of this experiment.

Total destruction of infected monolayers was observed with C. coli and Campylobacter strain 20/74, in about 3 to 4 days.

iv. Cytopathic changes observed in Vero and LLCMK$_2$ cells.

These cell lines did not attach any of the mucosalis strains and it was not possible, by light microscopy, to demonstrate the presence of intracellular organism (Chapters VI and VII). Cytopathic changes were not detected during the two week post-infection period except that a small percentage of the cells in the monolayers appeared to be 'swollen' during the first week of

of infection. There was no evidence that this change was progressive or resulted in the production of 'altered cells' as in other cell types.

C. coli and Campylobacter strain 20/74 produced complete destruction of the monolayers in less than 4 days post-inoculation.

Comment.

The above observations differed from those obtained following re-infection of once-infected Vero or $LLCMK_2$ cells (p. 101) in that the cytopathic changes were pronounced and the production of polykaryons in a large proportion of the monolayer took place around the 4th to 6th days after re-infection. The polykaryons contained 16 to 20 nuclei of different size and shape, and nuclear fragmentation was marked in degenerating fused cells (Figs. 36A-B In addition, 'altered cells' were present and often contained cytoplasmic vacuoles. Rapid destruction of the infected monolayers occurred 10 to 12 days post-inoculation.

v. Cytopathic changes observed in CEF.

Light microscopy of CEF cultures infected with mucosalis failed to show cytopathic changes and the monolayers remained attached to the glass for upto 10 days post-inoculation.

A marked destruction of CEF cells occurred within 2-4 days following infection with C. coli and Campylobacter strain 20/74.

Fig. 31A

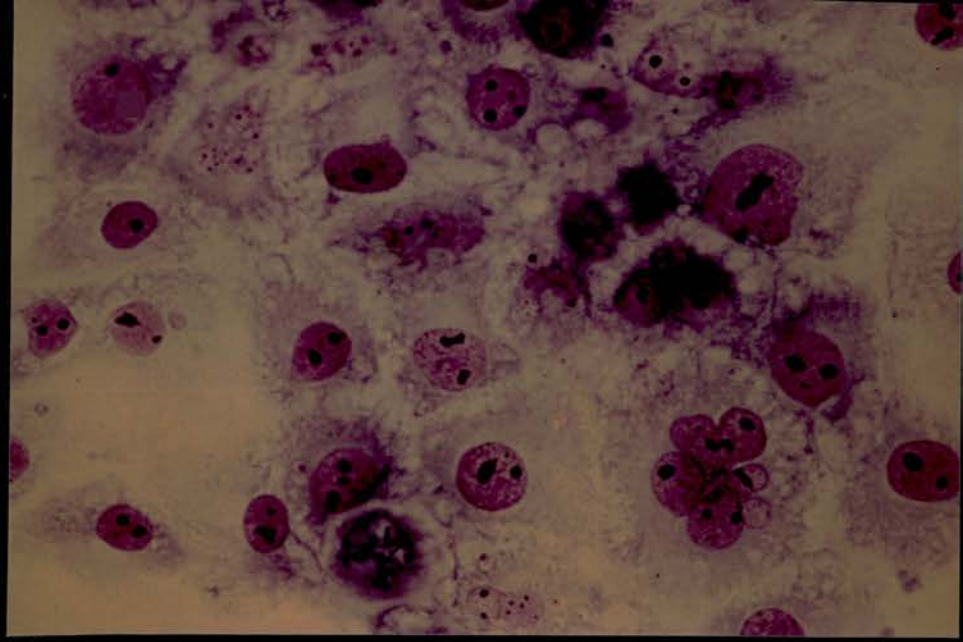
Two-day-old control monolayer of uninfected PK_{pi} cells. Giemsa X 160.

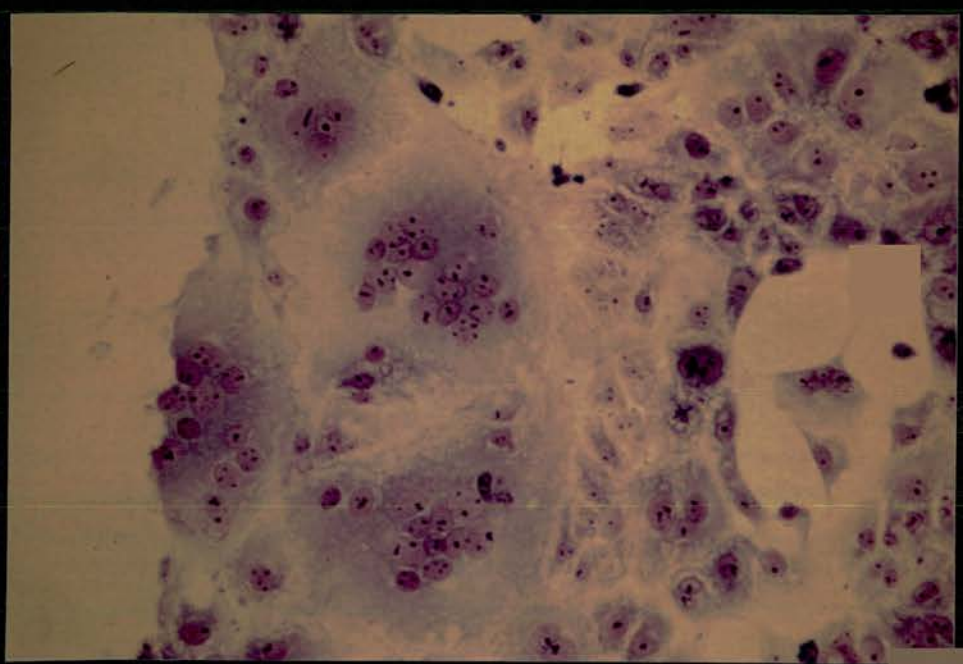
Fig. 31B

Monolayer of PK_{pi} cells infected 2 days previously with C. sputorum ss mucosalis, showing the formation of several, large, multinucleated syncytia. Note the absence of inclusion bodies. Giemsa X 160.

Fig. 32

Monolayer of BK cells, six days post-inoculation with C. sputorum ss mucosalis, showing a number of small foci of multi-nucleated cells and several swollen misshapen nuclei. Giemsa X 160.





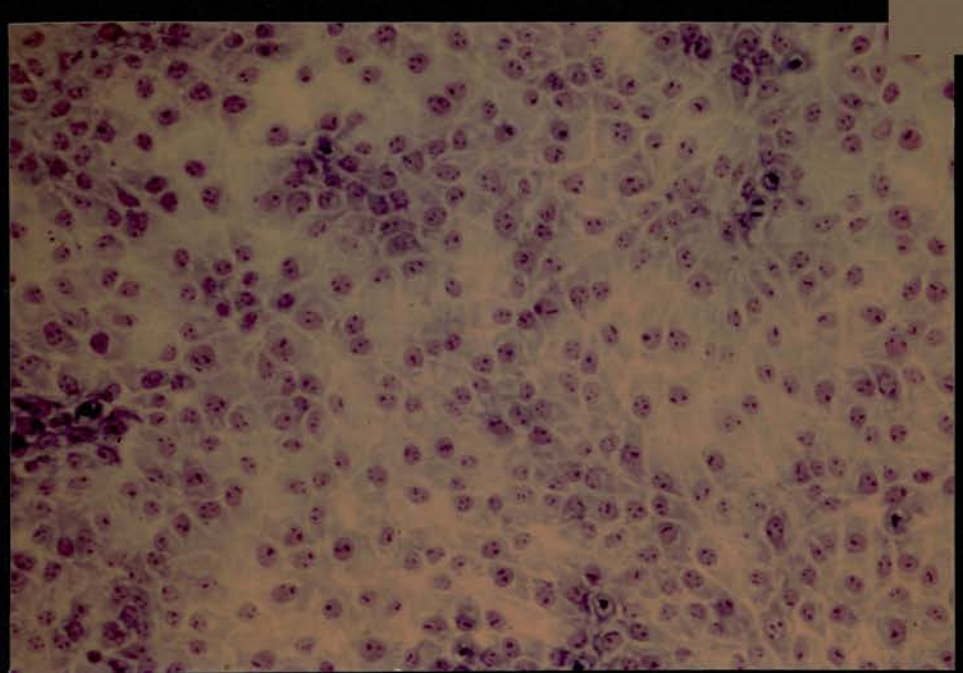


Fig. 33A

Unstained, uninfected 7-day-old BK cell control. X 64.

Fig. 33B

Unstained monolayer of BK cells 10 days after infection with mucosalis. There are numerous 'altered cells' and their markedly swollen cytoplasms are clearly seen. In some instances cell fusion has taken place between contiguous 'altered cells'. X 64.

It is emphasized that Figs. 33A and B are shown at the same magnification and clearly underline the marked enlargement of the 'altered cells' induced by mucosalis.

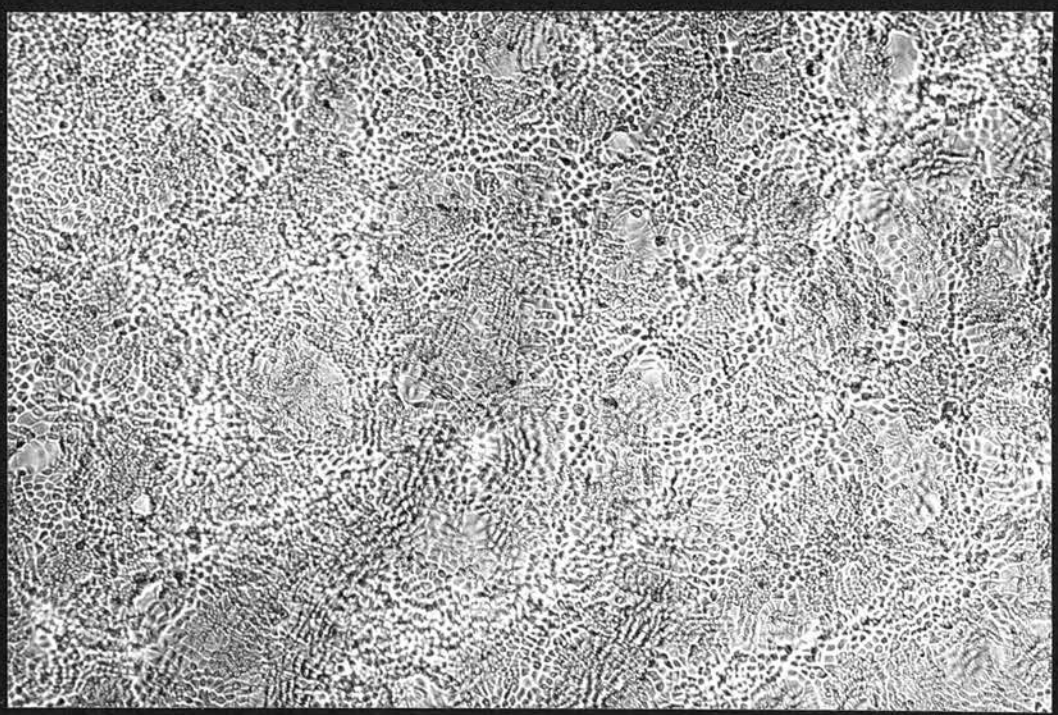
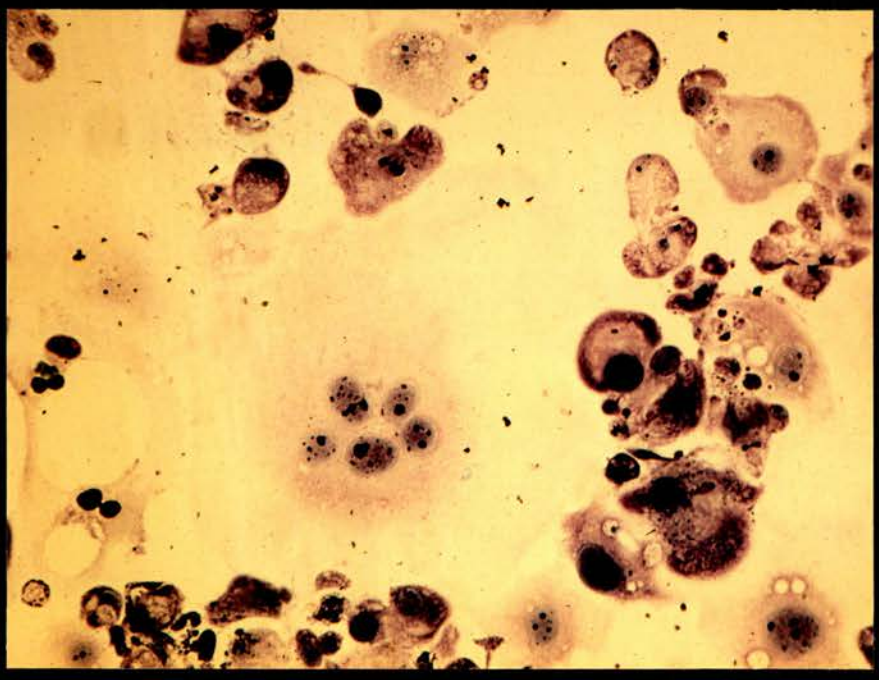


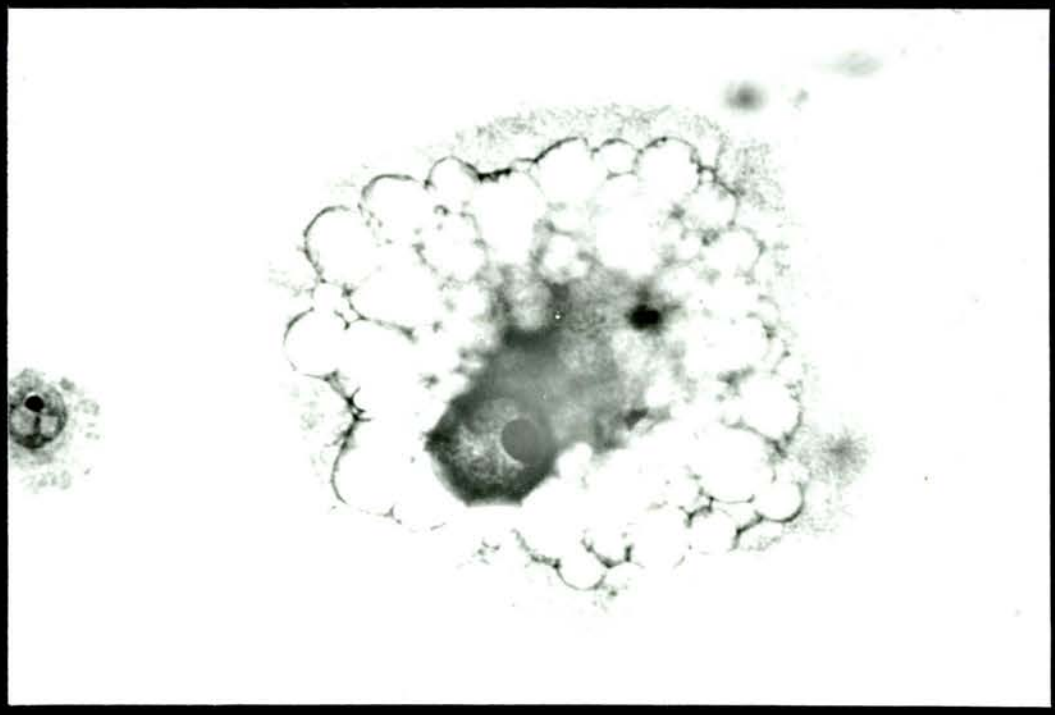
Fig. 34

C. sputorum ss mucosalis-induced CPE in a ten-day-old BK_{pi} monolayer, showing large multinucleate syncytia and several 'altered cells'. There is also evidence of cellular degeneration and cytoplasmic vacuolation. Giemsa X 160.



Figs. 35A and B

Degenerative changes observed in 'altered cells' after the third serial passage (63 days post-infection) of BK_{pi} cells infected with mucosalis. The CPE is characterized by massive cytoplasmic vacuolation. Giemsa X 160.



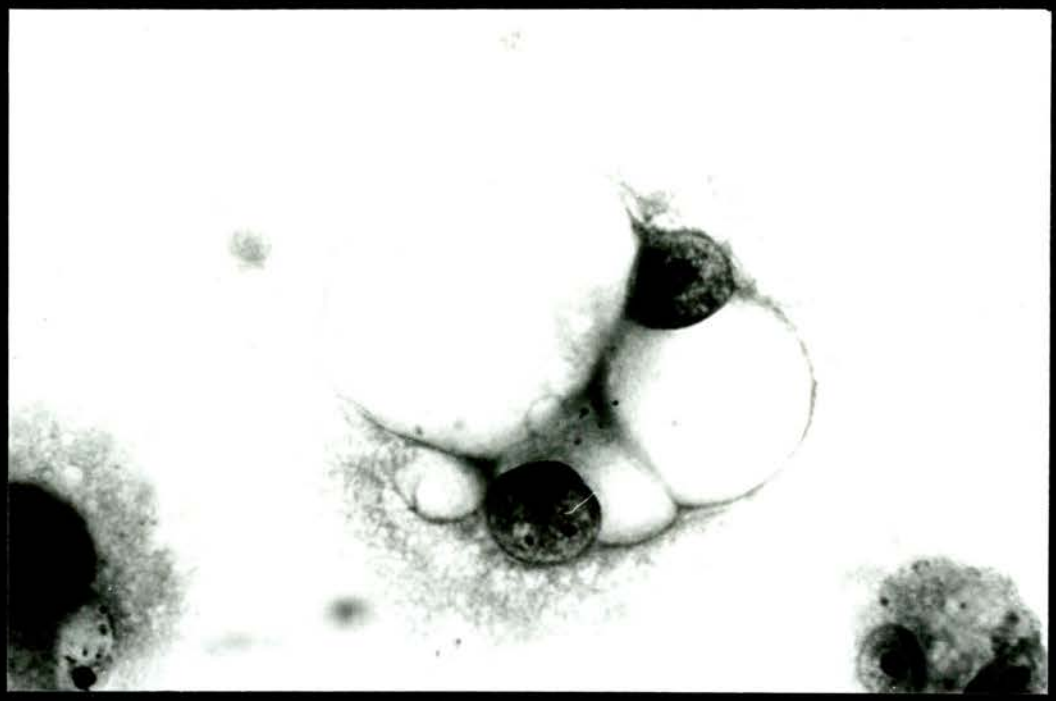
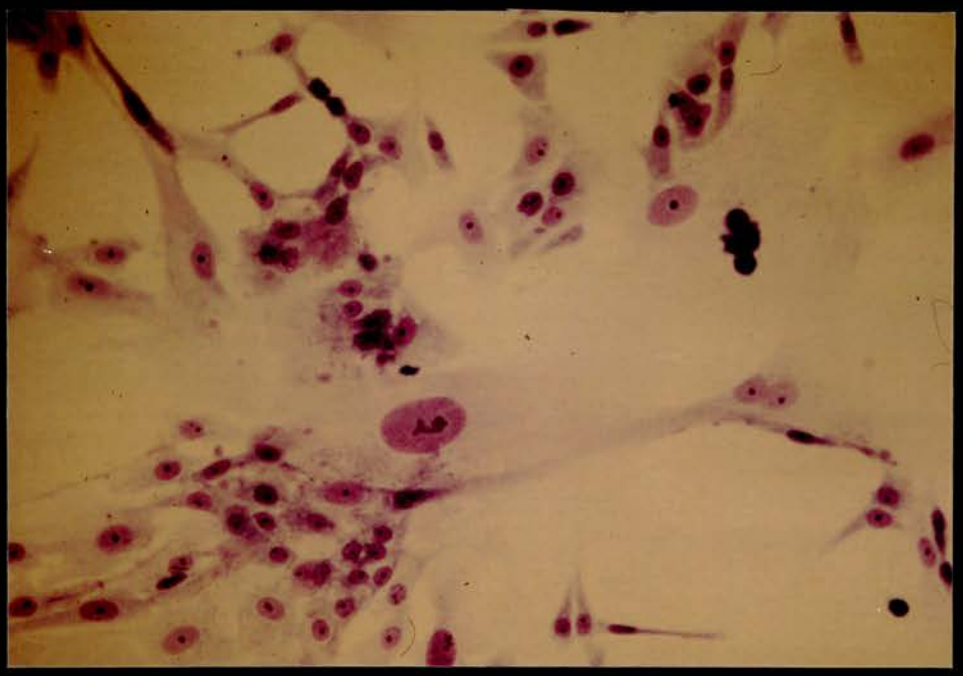


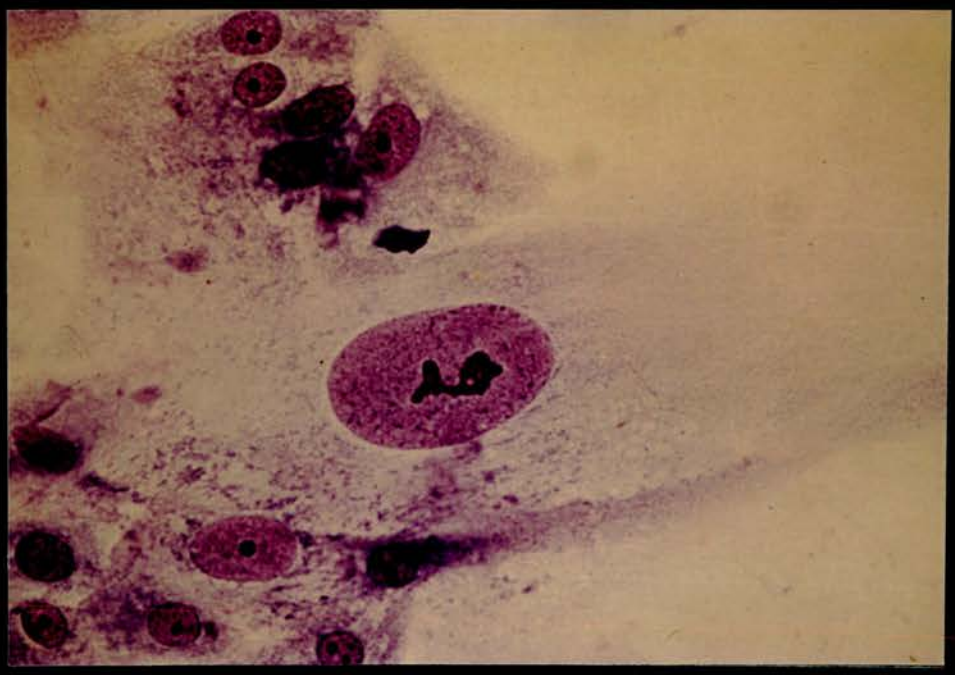
Fig. 36A

Effect of reinfection of Vero cells with C. sputorum ss mucosalis. Notice the sparse monolayer, evidence of cell fusion and occasional cells showing greatly enlarged nuclei. The extensive featureless cytoplasm surrounding these swollen nuclei is ill-defined. Giemsa X 160.

Fig. 36B

Detail of Fig. 35A showing greatly enlarged nucleus within an extensive sheet of cytoplasmic material. Giemsa X 500.





vi. Effects of bacteria-free filtrates of mucosalis on the production of CPE in cell cultures.

Coverslip preparations of PK, PK_{pi}, BK and BK_{pi} inoculated with bacteria-free filtrates obtained by passing a 24 or 48 hr CBA-MEM diphasic growth of mucosalis strain 253/72 through membranes of 0.45 or 0.22 μm A.P.D., remained normal and did not show any CPE during the 14 day period of this experiment.

B. EFFECT OF SERIAL-PASSAGE ON BK, BK_{pi}, OK_{pi}, BHK AND HeLa CELL CULTURES INFECTED WITH C. SPUTORUM SS MUCOSALIS.

a. Introduction.

Experimental evidence so far obtained has shown that nearly 30% of the cell population of BK, BK_{pi}, OK_{pi}, BHK and HeLa cells did not appear to contain any demonstrable intracellular C. sputorum ss mucosalis and that these cells also failed to undergo cellular abnormalities and remained apparently normal. Compared with pig kidney cell lines (PK and PK_{pi}) these cell cultures resisted the cellular destruction induced by the 'parasitic growth' of mucosalis organisms and a large number of infected cells remained attached to the glass. Therefore, it was considered important to investigate the behaviour of apparently normal cells present in cell cultures infected with mucosalis during serial passages.

b. Design of experiment.

The experimental procedures employed to infect suspensions of trypsinized cells of BK, BK_{pi}, OK_{pi}, BHK and HeLa cultures with mucosalis strain 253/72 and their subsequent serial passage have already been described on page 191. Infected and appropriate control cultures were incubated at 37°C, refed at weekly intervals with MEM containing 5% calf serum and split every 3 weeks. Approximately 1.5×10^6 cells/ml were seeded in medical flats and/or on coverslips. The presence of 'parasitic' mucosalis organisms in the supernatant fluids was examined prior to each subculture by the method described earlier (p.110). Methanol-fixed 2 to 3-day-old coverslip monolayers were stained by Giemsa's method and examined. Medical flats were routinely examined by light microscopy.

c. Results.

i. First passage (0-3 weeks): BK, BK_{pi}, OK_{pi}, BHK and HeLa cell cultures infected with mucosalis strain 253/72 showed cytopathic changes similar to those described in the previous experiment (p.218). All these cultures showed sparsely distributed islets of apparently healthy cells which were similar to the cells of uninfected control monolayers. Mucosalis organisms were isolated in large numbers from the supernatant fluids.

ii. Second passage (4-7 weeks): Despite extensive cellular changes 'altered cells', induced by mucosalis organisms, attached to the glass and formed confluent monolayers consisting mainly of large sheets of fused cells or cells

with markedly enlarged featureless cytoplasms. Apparently unaffected cells remained few in number and invariably occurred in small patches scattered between the 'altered cells'. At this stage, mucosalis could no longer be recovered from BHK and HeLa cell cultures although small numbers of organisms could be recovered from BK, BK_{pi} and OK_{pi} cells.

iii. Third passage (8-11 weeks):This phase of growth was characterised in early stages by rapid degenerative changes in the altered cells. Although affected cells failed to round up, they showed extensive nuclear fragmentation and foamy vacuolation (BK_{pi} and OK_{pi} cells) and were frequently found floating in the medium as 'jelly fish' like structures. Infected BHK and HeLa cells showed complete destruction of monolayers. Simultaneously, with these changes in the 'altered cells', there was rapid multiplication and a marked increase in the number of apparently healthy cells which tended to form islets of 100 or more cells. In general, bacteria could not be isolated from the cell culture fluids during this period.

iv. Fourth passage (12-15 weeks): Microscopic examination revealed almost complete absence of 'altered cells' although occasional slightly 'ballooned cells' were present. The monolayers of BK, BK_{pi}, and OK_{pi} were more or less confluent and cells rapidly grew without showing any cytopathic changes. Viable bacteria could not be isolated from these cell cultures.

v. Fifth passage (15-18 weeks): During this period the monolayers produced by 'apparently normal cells' of BK, BK_{pi} and OK_{pi} cells were indistinguishable from those of uninfected controls.

d. Comment.

The important factor that emerges from this experiment is the inability of mucosalis to infect the entire cell population. This phenomenon, although not unusual (e.g. virus infections), suggests that cell surface receptors for mucosalis adhesins may not be present in all cells of a population or that all cells are not equally susceptible to cytopathic changes by this organism.

Cell culture procedures employed in this experiment do not appear to be conducive to the persistence of 'parasitic growth' of mucosalis organisms. Weekly changing of the medium constantly drains the bacterial population and, at the same time, exposes the bacteria to the harmful effects of increased oxygen tension. However, even greater losses in the bacterial population can be expected during routine subculture of the infected monolayers. Such conventional procedures as repeated washing of the cells and low speed centrifugation of cell suspensions which leaves most bacteria suspended in the supernatant STV fluids must interfere with the persistence of 'parasitic growth' of mucosalis. It is believed that the repetition of these procedures may have been at least partly responsible for the disappearance of mucosalis infection in these cell cultures. It must be

stressed that cell cultures,by themselves, require regular feeding and maintenance for satisfactory growth and a compromise appears to be unlikely in such situations.

II. SOME OBSERVATIONS ON 'ALTERED CELLS' INDUCED BY C. SPUTORUM SS MUCOSALIS.

A. CYTOPATHIC CHANGES IN PK_{pi} CELL CULTURES TREATED WITH NDV-ANTISERUM.

a. Introduction.

Three of the cell lines (PK_{pi}, BK_{pi} and OK_{pi}) used in this study have been persistently infected for many years with a defective strain of NDV, and haemadsorption and immunofluorescence staining techniques have confirmed that over 95% of the cell population are infected (Fraser et al., 1976). Although confluent monolayers of morphologically normal cells are produced by these carrier cultures, stained preparations usually show a few, small foci of fused cells and, sometimes, intracytoplasmic inclusions.

These chronically infected cell lines are of particular interest to this current work since maturation and release of the persistent membrane-forming virus (NDV) is invariably associated with thickening and other changes of the plasmalemma which might influence the attachment

and penetration of C. sputorum ss mucosalis. Apart from the fact that the response of such cells to infection with mucosalis might differ considerably from that of other types of cell cultures, it was thought that their altered host cell metabolism might have some bearing on possible multiplication and release of progeny bacteria from mucosalis-infected cell cultures. The characteristics of these 'carrier' cell lines have been reported fully by Edwards (1972) and Louza (1977).

It is evident from the results obtained so far in this study that mucosalis infections of normal and 'carrier' cell cultures showed considerable variation in the degree of bacterial attachment and the type of CPE produced. For example, PK_{pi} cells showed greater bacterial attachment and more extensive cell fusion compared with moderate attachment and complete destruction of parent PK cells. Not only is it unusual to find that a bacterial infection is capable of inducing multinucleate polykaryon formation in cell cultures, but it is also of interest that mucosalis, which is associated with proliferative tissue changes in PIA, produces different types of CPE in PK cells depending on the presence or absence of the 'carrier' virus. In this connection it may be significant that the persistent virus (NDV) is one of the large groups of 'membrane-forming' viruses whose structural proteins assemble underneath the plasmalemma prior to the release of mature progeny virus by budding from the affected cell.

Earlier work by Fraser *et al.*, (1976) has shown that incorporation of NDV-antiserum in the medium promptly inhibits viral synthesis as well as cell-fusion, and an experiment was therefore designed to exploit this property and to study the type of CPE produced by *mucosalis* organisms in 'carrier' lines maintained in medium containing NDV antiserum. The PK_{pi} cell line was chosen in preference to BK_{pi}, because the latter tended to have more syncytia and occasionally showed intranuclear as well as intracytoplasmic inclusions. The use of OK_{pi} line was discounted because cultures of the uninfected parent line are no longer available. Further, the fact that *mucosalis*-infected PK_{pi} cells produced extensive cell fusion compared to other cell types, provided an ideal opportunity to examine cell fusion as a function of *mucosalis* infection.

b. Design of experiment.

PK_{pi} cells were trypsinized and subcultures grown in the presence of 4% anti-NDV serum prepared in rabbits (anti-haemagglutination titre, 512). After 5 days' incubation, the monolayers were subcultured, again incorporating 4% NDV anti-serum in the medium, and were used as 24 hr-old coverslip preparations for infection with the *mucosalis* strain 253/72. This was achieved by replacing the supernatant fluid with 1 ml of bacterial suspension in MEM growth medium containing 4% NDV antiserum. The methods of preparing the bacterial suspension and infecting the cell cultures were similar to those described previously (Chapter IV, p. 95). Coverslip cultures of

infected and uninfected cells grown in the presence of rabbit anti-NDV serum were removed at daily intervals, rinsed in PBS, fixed in methanol and then stained by Giemsa's method.

c. Results.

Despite pretreatment with NDV-antiserum, PK_{pi} cells infected with mucosalis cells showed extensive cell fusion (3rd day), polykaryon formation (4th day) and detachment from the glass (5th day), which is very similar to the results obtained earlier without NDV-antiserum. It should be emphasized however that the antiserum completely prevented the formation of small NDV-induced syncytia in control cultures uninoculated with mucosalis and that the cells remained firmly attached to the glass. This clearly indicates that cell fusion and polykaryon formation observed in PK_{pi} cells inoculated with mucosalis is primarily due to the bacterial infection.

d. Comment.

The presence of extensive cell fusion in PK_{pi} cells pretreated with NDV antiserum to inhibit viral replication, clearly indicates that the formation of large multi-nucleated polykaryons is associated with mucosalis infection and is not due to the 'carrier' virus. A pilot test has indicated that mucosalis infection of PK_{pi} cells does not enhance the replication of NDV and further evidence that a virus is not essential for cell fusion is provided by the observations that mucosalis organisms are able to produce similar CPE in other cell culture systems whether or not they are persistently

infected with NDV.

There appears to be no parallel example in which a bacterium is associated with the induction of extensive cell fusion. Although considerable progress has been made in recent years towards understanding the mechanisms of viral induced cell fusion (Poste and Waterson, 1975), little is known about the nature of mucosalis-induced cell fusion and much further work is urgently required to explain this phenomenon.

B. HAEMADSORPTION BY 'ALTERED CELLS' IN C. SPUTORUM SS MUCOSALIS-INFECTED CULTURES.

a. Introduction.

It is emphasized that three of the cell lines (PK_{pi}, BK_{pi} and OK_{pi}) used in this investigation have been persistently infected for approximately 20 years with a defective strain of Newcastle disease virus and, as a result, almost all the cells (over 95%) carry the virus and have altered membranes which readily adsorb avian and guinea-pig erythrocytes (Fraser et al., 1976).

b. Design of experiment.

In view of the different responses of PK_{pi}, BK_{pi} and OK_{pi} cells to mucosalis infection, the ability of 'altered cells' of all these lines to haemadsorb fowl and guinea-pig red blood cells (RBCs) was examined at various intervals following infection. Coverslip preparations of infected PK_{pi} cells were examined on the 3rd and 4th days, while those of BK_{pi} and OK_{pi} were examined on the 7th and 14th days post-inoculation. In a separate series of experiments, 'altered cells' of infected BK_{pi}

and OK_{pi} obtained after the 2nd and 3rd serial passages were also tested for haemadsorption.

These coverslip cultures, and their appropriate uninfected controls, were thoroughly rinsed in PBS and each was overlaid with 1 ml of a 0.5% suspension of freshly drawn guinea-pig or fowl erythrocytes. After incubation at 37°C for 15 min, the monolayers were thoroughly rinsed in several changes of warm PBS, fixed in methanol and stained with Giemsa.

c. Results.

Microscopic examination of stained coverslip preparations confirmed the presence of haemadsorption by guinea-pig and fowl erythrocytes to the surfaces of mucosalis-induced 'altered cells' of PK_{pi}, BK_{pi} and OK_{pi} lines. The degree of haemadsorption was similar to that of control cultures uninfected with mucosalis.

d. Comment.

It is interesting to note that infection with mucosalis did not affect the ability of 'carrier' lines to haemadsorb guinea-pig and chicken erythrocytes. The fact that haemadsorption also occurred on the surface of the thin and featureless cytoplasms of 'altered cells' suggests that these abnormal structures develop from NDV-permissive cells only or from a mixture of non-permissive and permissive cell types. Since none of the 'altered cells' failed to show haemadsorption it is possible that the non-permissive cells (5%) in the NDV carrier cultures cannot be induced to produce 'altered cells' by mucosalis infections. The effects of mucosalis

on the replication and release of virus from persistently infected cells are matters for further investigations.

C. BINDING OF CON-A TO THE 'ALTERED CELLS'.

a. Introduction.

The results obtained so far have indicated that PK_{pi} and BK cells carry specific carbohydrate receptors on their surfaces which bind Con-A, and that pretreatment with Con-A antagonist methyl α-D-mannopyranoside completely blocks the binding of the lectin, Con-A.

'Altered cells' induced by C. sputorum ss mucosalis are morphologically very different from uninfected parent cells and, in some lines, the predominant feature is the presence of 'swollen' cells with a thin and featureless cytoplasm. It was therefore of interest to ascertain whether these Con-A specific carbohydrate complexes remained on the cell surfaces of 'altered cells'.

b. Design of experiment.

Monolayers of PK_{pi} and BK cells infected with mucosalis organisms were subcultured after treatment with STV on the 3rd and 8th days post-infection, respectively. Coverslip preparations of 'altered cells' thus obtained (1- 2- 3 and 4 days post-infection) together with appropriate uninfected controls were exposed to fluorochrome conjugated Con-A suspension. Occasionally, 'altered cells' obtained during the 2nd and 3rd blind passages of BK cells infected with mucosalis organisms, were similarly examined after Con-A treatment. The specificity of Con-A binding was checked by pretreatment

with methyl α-D-mannopyranoside as described in Chapter VI (p.171).

c. Results.

The fact that 'altered' PK_{pi} and BK cells, unlike the uninfected controls, were unable to bind Con-A, indicated the possible absence of specific carbohydrate complexes on their cell surfaces. On the other hand it was noticed that BK cells obtained from the 2nd and 3rd blind passages contained occasional islets of cells that specifically bound Con-A, and that the reaction was selectively blocked by pretreatment with methyl α-D-mannopyranoside. The production of morphologically normal islets of cells during blind passages of infected BK cells has been noted previously and might account for the selective binding of Con-A observed above.

d. Comment.

The absence of Con-A binding to 'altered' PK_{pi} and BK cells, unlike the binding present in non-infected cells, suggests that the cytopathic effects induced by mucosalis were also associated with structural changes on the cell surface. Although similar variations in the binding of lectins in virus-transformed cell cultures have been reported by other workers (Nicolson, 1974), considerable further work is clearly necessary in order to explain the mechanisms involved in the mucosalis induced alterations of the surface membranes.

D. ATTACHMENT OF C. SPUTORUM SS MUCOSALIS TO 'ALTERED CELLS.

a. Introduction.

C. sputorum ss mucosalis adheres selectively to cell surfaces and, in cultures of permissive cells, attachment reaches a maximum around 4 to 6 hr but is only present for 12 hr post-inoculation. Experimental evidence has indicated that the adhesive properties of this organism are multifactorial and that they are influenced by bacterial motility and the nature of the surface receptors present on bacterial and host cells (Chapter VI).

'Altered cells' induced by mucosalis failed to 'round-up' when trypsinized with STV solution and remained as large amorphous structures. This unusual behaviour of 'altered cells', along with their inability to bind Con-A, suggested that functional changes had taken place on the cell surfaces. It was therefore decided to examine the adhesive properties of 'altered cells' for mucosalis organisms since attachment reflects the function of the cell membrane and of the host cell surface receptors in particular.

b. Design of experiment.

Coverslip preparations of 'altered cells' of PK_{pi} and BK cells were obtained by the method described in the previous experiment. Two-day-old monolayers were rinsed in three changes of PBS and overlaid with 1 ml of a suspension of mucosalis strain 253/72, in MEM. These, together with a number of appropriate inoculated, unaltered cell cultures, were incubated for 2, 4, 6, 8

and 24 hr at 37°C, rinsed thrice in warm PBS to remove unattached bacteria, stained by Giemsa's method and examined.

Also examined for bacterial attachment were coverslip preparations of 'altered cells' obtained during the 2nd, 3rd and 4th blind passages of BK cells infected with mucosalis strain 253/72. In a further series of experiments, attachment of formol-saline and air-killed mucosalis organisms to 'altered cells' was attempted.

c. Results.

Giemsa stained preparations of 'altered' PK_{pi} and BK cells exposed to viable and killed mucosalis organisms showed complete absence of bacterial attachment. A small percentage of morphologically normal cells in the passaged BK cultures revealed occasional attachment with viable and air-killed bacteria but not with formol-saline inactivated organisms. By comparison, extensive bacterial attachment was observed in the control cultures during the first 8 hr, but not at 24 hr, post-inoculation.

d. Comment.

The absence of bacterial attachment on re-infected 'altered cells' confirms that the cytopathic effects induced by mucosalis are associated with the loss of mucosalis-specific receptors on the cell surfaces. The precise nature of the receptors involved in mucosalis attachment has not yet been determined but the above results, together with the earlier observations on the selective binding of lectin Con-A, indicate that these effects could be complex.

E. ATTEMPTS TO ESTABLISH A LINE OF 'ALTERED CELLS'.

a. Introduction.

The results obtained so far clearly indicate that there is marked variation in the response of different types of cell cultures to C. sputorum ss mucosalis infection. This includes their ability to support 'parasitic growth' and undergo cytopathic changes resulting in the induction of 'altered cells'. Although the precise mechanisms by which mucosalis achieves these effects are not fully understood the production, in vitro, of these enlarged 'altered cells' could be of significance since they may bear some relation to the tissue changes observed in field cases of PIA.

b. Design of experiment.

i. Selection of cell types.

It is clear from the experiments involving serially passaged BK, BK_{pi} and OK_{pi} cells and, to a limited extent, HeLa and BHK cells, that cultures infected with mucosalis organisms usually contained some 30% of cells which appeared to be free of demonstrable intracellular bacteria. These cells also failed to undergo the cellular changes that were characteristic of in vitro mucosalis infection. Clusters of these morphologically normal cells gradually increased in number during serial passages of infected monolayers until they completely replaced the mucosalis-induced 'altered cells'.

In contrast, PK_{pi} cells grown under similar experimental conditions showed cytopathic changes, but the affected cells were not replaced by apparently healthy cells.

Because of this absence of residual normal cells it was decided to employ PK_{pi} cells in attempts to establish a line of 'altered cells'.

ii. Cell culture methods.

The technique of infecting suspension of trypsinized PK_{pi} cells with mucosalis strain 253/72 has already been described (Chapter IV). After 6 hr of incubation at 37°C, all unattached bacteria were removed by thoroughly rinsing the monolayers with sterile PBS. The cultures were then refed and incubation continued. Rinsing, refeeding and incubation were repeated at 24 and 48 hr to remove extracellular bacteria and bacterial metabolites. Infected cell cultures were trypsinized on the third day post-inoculation and the 'altered cells' (2×10^6 cells/ml) allowed to form monolayers in the presence of MEM containing a higher concentration of calf or foetal calf serum (20-30%). Medical flats seeded with these cells were filled completely with this modified medium in order to eliminate the air-atmosphere and thereby provide possible alternative growth conditions.

c. Results.

Light microscopy of medical flats seeded with 'altered' PK_{pi} cells and incubated for 24 hr at 37°C in enriched modified MEM showed that the 'altered cells' had attached to the glass and formed a thin sheet consisting essentially of massive plaques of fused cells with featureless cytoplasms. Although further incubation resulted in an increase in granularity of the featureless cytoplasms, there was no evidence of cellular multiplication. In cultures maintained in the normal volume of

medium (10 ml/4 oz bottle), all the cells remained attached to the glass for 5-6 days whereas in medical flats, filled completely with medium, total destruction of the monolayers occurred in less than 3 days.

Examination of Giemsa stained coverslip preparations confirmed the gradual increased granularity of the cytoplasms of 'altered cells' during the first 3 days of incubation, followed by marked cytoplasmic vacuolation and destruction of the monolayer. Nuclear changes were seen after 72 hr and included extensive fragmentation with, or without, the formation of large clusters of vacuoles. There was no evidence of 'altered cells' being replaced by apparently normal cells, as was the case in other types of cell cultures inoculated with mucosalis.

d. Comment.

These findings confirm that cytopathic changes occur readily in PK_{pi} cells infected with mucosalis, and that the addition of 20-30% calf or foetal calf serum to the medium favours prolonged attachment and survival of the 'altered cells', but does not enable them to multiply.

III. CYTOPATHIC CHANGES PRODUCED BY ULTRASONIC DISINTEGRATES OF C. SPUTORUM SS MUCOSALIS.

a. Introduction.

The ability of C. sputorum ss mucosalis to induce cytopathic changes in different types of cell cultures is an important observation and, although the mechanisms involved are not fully understood, the present findings have proved helpful in attempting to clarify the issue. These can be summarised as follows:

i. Bacteria-free filtrates obtained from CBA-MEM diphasic growth of mucosalis failed to induce CPE.

ii. Despite prolonged 'parasitic' association, this non-obligate fastidious organism was unable to invade all the cells of the monolayers. Some 30% of the cell population remained free of intracellular bacteria or particulate bacterial antigens.

iii. Serial passage of infected monolayers resulted in gradual elimination of 'parasitic growth' of mucosalis organisms and simultaneous replacement of mucosalis-induced 'altered cells' by apparently healthy bacteria-free cells.

iv. These observations clearly suggest that the production of CPE in cell cultures is associated with either intracellular multiplication or with the intracellular location of non-viable mucosalis

rather than the effects of bacterial toxins, metabolites or 'parasitic growth'. The design of some of the experiments undertaken, including the use of surface growth of mucosalis and infection of trypsinized cells followed by regular rinsing and refeeding procedures, were aimed to minimise the possible influence of bacterial toxins, metabolites and 'parasitic growth' on the production of CPE. Having obtained evidence of bacterial intracellular multiplication by viable counts and by immunofluorescence staining procedures, it was considered important to ascertain whether ultrasonic disintegrates of mucosalis also had the ability to induce cytopathic effects similar to those produced by viable organisms.

b. Design of experiment.

i. Preparation of ultrasonic disintegrates.

The surface growth of a 24 hr-old culture of C. sputorum ss mucosalis (strain 253/72) on CBA plates was harvested in sterile PBS (approximately 10 x 10^8 bacteria/ml) and subjected to ultrasonic disintegration in a MSE ultrasonic disintegrator (Measuring and Scientific Equipments, London) at 18,000-20,000 c.p.s. for 10-15 min held on ice. The sonciates were examined for the presence of undisrupted residual bacteria in Giemsa stained preparations and were then incubated overnight at 37°C to kill any viable mucosalis organisms. Both filtered and unfiltered material were used as inocula, the former being prepared by passing the sonicate through a Millipore membrane filter of 1.0 μm, 0.45 μm and 0.22 μm A.P.D., respectively.

ii. Inoculation of cell cultures with sonicates.

One-day-old coverslip cultures of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, HeLa, BHK, DK, Vero and $LLCMK_2$ cells were overlaid with 1 ml of MEM containing 5% calf serum and 1:10 (V/V) dilution of each of the above ultrasonic fractions of mucosalis. These and a number of appropriate unexposed control monolayers were incubated at 37°C and examined daily after staining by Giemsa's method. In selected experiments, coverslip preparations of trypsinized PK_{pi} and BK cells inoculated with different mucosalis sonicates were incubated overnight at 37°C, rinsed in 3 changes of PBS to remove unbound bacterial disintegrates and returned to the incubator after being refed with fresh MEM. These, and their appropriate controls, were processed in a similar manner to the preformed monolayers (vide supra).

iii. Immunofluorescence staining of PK_{pi} and BK cells inoculated with sonicates.

In selected experiments, several one-day-old coverslip cultures of PK_{pi} and BK cells were overlaid with 1 ml of each ultrasonic fraction of mucosalis preparations. These and a number of unexposed control monolayers were incubated at 37°C and examined daily for 5 days post-inoculation. After formaldehyde or acetone fixation, coverslips were stained with rabbit anti-mucosalis serum and examined.

c. Results.

i. Crude mucosalis sonicates.

Microscopic examination of Giemsa-stained preformed monolayers of PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK, HeLa and DK cells inoculated with crude sonicates of mucosalis showed marked attachment of disrupted bacterial fragments to the surfaces of almost all cells. Aggregation of these sonicated particles did not increase with further incubation, and attachment did not occur on cell-free areas of the coverslip preparations. Because of the marked 'coating' and poor staining reaction of mucosalis sonicates on cell surfaces, it was difficult to determine whether or not phagocytosis of this material had taken place. However many cells showed vacuolation and these vacuoles contained similar material to that observed on the cell surface. Surface accumulation of sonicated bacterial fragments was quickly followed by the development of cytopathic changes and it was interesting to note that, of all the cell lines examined, PK, and PK_{pi} cells were the first to show CPE, as had been the case in cell cultures infected with viable mucosalis organisms. On the second day of incubation PK monolayers showed large areas of rounded cells followed by complete destruction of the cell sheet on the 3rd day. In PK_{pi} cell cultures the cellular changes included extensive cell fusion within 48 hr followed by total destruction of the monolayer on the third day. Although the type of cell fusion observed in PK_{pi} cells was similar to that described in an earlier experiment (p.215), the cell changes were generally more extensive

and were invariably associated with marked nucleorrhexis and rapid degenerative changes.

Other cell types, including BK, BK_{pi}, PK_{pi}, HeLa and BHK, appeared to be more susceptible to the action of mucosalis sonicates than to viable organisms and, significantly, showed an early 'ballooning effect' on the 2nd day followed by marked cell fusion on the 3rd to 4th day post-inoculation. In addition, a large percentage of cells showed nucleorrhexis while rounding up of the cells was particularly evident in the BHK line. In BK_{pi} and OK_{pi} cells there was evidence of increased cytoplasmic vacuolation. Detachment of cells from the glass was rapid and in all of these cell lines total destruction of the monolayers occurred within 4-5 days.

Cultures of DK cells were also capable of attaching sonicated bacteria to their surfaces but, in contrast to the other lines mentioned above, were less readily affected by the inocula and did not show marked cellular changes. Although examination of stained DK cultures indicated that the apparently normal monolayers contained approximately 10-20% of pyknotic and rounded cells, the only abnormality observed 4 days post-inoculation was the presence of a patchy type of sub-confluent growth compared with the uniformly compact cell sheets which characterised the uninoculated control monolayers.

Vero and $LLCMK_2$ cells were unusual in that they showed negligible surface aggregation of bacterial sonicates, remained firmly attached to the glass and failed to show any cytopathic change throughout the course of the experiment.

ii. Filtered (1.0 μm and 0.45 μm) sonicates.

Monolayers treated with mucosalis sonicates (passed through membrane filters of 1.0 μm and 0.45 μm A.P.D., respectively) showed no evidence of cell-bound aggregates resembling those obtained with the crude sonicate (vide supra). However, after 24 hr of incubation the cell surfaces appeared 'foggy' and seemed to be coated with fine particulate matter that was also present in the ultrasonic filtrate. No other abnormalities were seen and it was interesting, therefore, to find that cytopathic changes developed that closely resembled those obtained in cell cultures inoculated with crude bacterial sonicates. It should be emphasized that Vero and $LLCMK_2$ cells did not show the fine particulate surface coating, and did not produce cellular abnormalities.

iii. Filtered (0.22 μm) sonicates.

Examination of Giemsa-stained coverslips of all types of cell cultures inoculated with 0.22 μm filtrates showed that the monolayers consisted of apparently healthy cells without evidence of CPE.

Throughout the period of examination, all uninoculated control monolayers remained attached to the glass and did not show any cellular abnormality.

iv. Examination of trypsinized PK_{pi} and BK cells inoculated with mucosalis sonicates.

Suspensions of trypsinized PK_{pi} and BK cells inoculated with either crude or filtered mucosalis sonicate fractions grew well and attached to the glass after overnight incubation at 37°C, but examination of Giemsa-stained cultures showed that the cell sheets remained

sub-confluent and had a patchy appearance compared with the uninoculated control monolayers.

With 'crude' and filtered (1.0 μm and 0.45 μm) sonicates, the distribution of particulate material on the cell surfaces and the nature of the induced cytopathic changes were very similar to those obtained earlier with preformed monolayers. In this present experiment however, the greater part of the monolayer became detached from the glass within 3 days. In contrast, cell suspensions inoculated with 0.22 μm filtrates formed confluent monolayers which remained attached to the glass for 7 days or longer, and did not show CPE.

v. Demonstration, by immunofluorescence staining, of mucosalis antigens in cell cultures inoculated with sonicates.

Examination of formaldehyde-fixed monolayers revealed specific fluorescence on the cell surfaces of PK_{pi} and BK cells upto 4 to 5 days post-inoculation. Monolayers inoculated with crude and 1 μm filtered sonicates were heavily 'coated' with large particles of brightly fluorescing antigenic material whereas those treated with 0.45 μm filtrates showed the presence of finely granular fluorescing material distributed uniformly on the cell surfaces. Cell cultures inoculated with 0.22 μm filtrates failed to show any fluorescence suggesting that such preparations did not contain antigens detectable with the antiserum employed (Fig. 37).

Fig. 37

A formaldehyde-fixed monolayer of BK cells inoculated 3 days previously with ultrasonic filtrates (1.0 μm A.P.D.) of C. sputorum ss mucosalis and examined by immunofluorescence staining. The particulate bacterial antigens fluoresce brightly and are widely distributed over the surfaces of the affected cells. X 172.

Acetone-fixed monolayers showed a similar staining reaction although the degree of fluorescence was generally less intense. Because of the intense 'coating' of the cells by the bacterial sonicates it was not possible to differentiate clearly between intracellular and extracellular antigens.

d. Comment.

The findings of these experiments are of interest in that they demonstrate, i) the ability of sonicated bacterial fragments to adhere selectively to certain types of cell cultures, ii) that ultrasonic disintegrates of mucosalis, both before and after passage through membrane filters of 1.0 and 0.45 μm APD, are capable of producing characteristic CPE and appear to be more harmful to the cells than are intact viable/organisms or even the effect of 'parasitic' (mucosalis) multiplication and iii) that 0.22 μm membrane filtrates are incapable of inducing CPE in permissive cells.

The findings also suggest that peptidoglycan and other bacterial cell wall components may be involved in the production of CPE in cell cultures since the 0.22 μm filtrates, which do not appear to affect cells, are unlikely to contain bacterial cell wall macromolecular components although they may not be completely free of degraded cell wall components. Thus, bacterial nucleic acids, which are likely to form a major component of 0.22 μm filtrates, appear to be non-toxic to cell cultures and incapable of producing CPE.

It is emphasized however, that very little is known about the chemical composition of mucosalis cells and their ultrasonicated fractions, and that the role of bacterial macromolecular components, as suggested by these experiments must await confirmation. However, the assumptions made in this chapter are supported by the earlier immunofluorescence and electron microscopic studies of mucosalis-infected monolayers which showed, first, the presence of specific intracellular bacterial antigen and, secondly, that intracellular structures morphologically indistinguishable from those of bacterial cell walls are regularly observed in electron micrographs of infected monolayers. Further studies involving chemically defined fractions of mucosalis are required in order to obtain a better understanding of the unusual cytopathic changes produced by this organism.

DISCUSSION.

Little information is available regarding the occurrence of C. sputorum ss mucosalis in domestic animals, apart from its association with PIA and related enteropathies in the pig. It would appear likely that the pig is the natural or primary host of this organism. However, in this present study, the results obtained from infection by mucosalis of no fewer than twelve types of cell cultures (PPK, PK, PK_{pi}, BK, BK_{pi}, OK_{pi}, BHK, HeLa, DK, Vero, $LLCMK_2$ and CEF) from various species of animals clearly show

that the organism attaches, is phagocytosed by and produces unusual cytopathic effects in a number of cell types with or without evidence of 'parasitic' multiplication. The nature of mucosalis-cell culture interactions is of interest and may indicate the mechanisms by which cell alterations are produced in vivo.

It is well known that many animal viruses are able to replicate in vitro although they often fail to produce a CPE in infected monolayers. In Bacteriology, however, there is no parallel situation since there are no reports of selective growth of pathogenic bacteria without the production of CPE or destruction of the infected cell cultures (Chapter I). The destructive changes induced by bacteria are largely due to i) their intrinsic ability for extracellular growth in cell culture media, and ii) phagocytosis by the host cell since intracellular bacteria appear to exert toxic effects and usually cause complete destruction of the infected monolayers. Such extracellular growth may be eliminated by antibiotic treatment but invariably fails to prevent the degenerative cellular process induced by the phagocytosed organisms.

In this present study the behaviour of mucosalis in twelve different types of cell cultures presents an entirely different picture from that obtained by workers with other pathogenic bacteria. The most interesting features are i) the ability of trypsinized cells cultivated with mucosalis to form confluent monolayers with or without prolonged parasitic multiplication of the

organism, ii) the survival of some 20-30% of healthy cells in the infected monolayers despite the presence extracellularly of mucosalis organisms, iii) the production of various cellular responses in the infected cell cultures ranging from total cell destruction to absence of CPE, and iv) the gradual elimination of 'parasitic multiplication' and replacement of 'altered cells' induced by mucosalis by apparently healthy cells during blind serial passages. It is emphasized that the available literature on infections of cell cultures with other species of bacteria does not show a situation comparable with that of mucosalis.

Although a great deal of additional information is required in order to understand fully the nature, mechanisms involved and significance of this unusual 'parasitic growth' and the CPE produced, the findings of this study are not only of considerable fundamental importance but may also help to explain the pathogenesis of PIA. The responses of cell cultures to infection with mucosalis can be broadly grouped as follows:

i. cell cultures that show 'parasitic multiplication' and rapid CPE with total destruction of infected monolayers. These include PPK, PK and PK_{pi} cells.

ii. cell cultures that are less readily destroyed by mucosalis infection and give rise to the production of markedly enlarged 'altered cells'. These monolayers not only remained attached to the glass for long periods but contain about 30% of apparently

healthy cells in addition to the 'altered cells'. Such cultures include BK, BK_{pi}, OK_{pi}, BHK and HeLa cells.

iii. cell cultures, including Vero and $LLCMK_2$ lines, that are 'initially' refractory to 'parasitic growth' and the production of CPE but which, on reinfection, behave like those in group ii, except that there is ultimately total loss of the monolayers.

iv. cell cultures that normally support parasitic multiplication but fail to show any CPE, of which DK cells is an example,

v. cell cultures that fail completely to support parasitic multiplication and do not induce CPE, even after super-infection e.g. primary cultures of CEF.

It is evident from the above classification that there is considerable variation in the degree of susceptibility to infection and the production of CPE among different types of cell cultures, and these findings clearly emphasize the complex nature of mucosalis-cell interactions in vitro. No single explanation is possible at present for all of these events although it must be emphasized that these cytopathic changes cannot be induced by formol-saline inactivated or air-killed bacteria, or by bacteria-free filtrates obtained from mucosalis diphasic growth. However, it seems that these cellular changes are invariably associated with infection by viable mucosalis organisms or their ultrasonic disintegrates.

BK_{pi} and OK_{pi} cultures persistently infected with NDV showed marked adsorption of guinea-pig and fowl erythrocytes against the background of thin featureless cytoplasms, but the degree of adsorption was similar to that obtained with uninfected parent cells. Further attempts to characterise these mucosalis-induced 'altered cells' are essential for an understanding of the mechanisms involved.

The nature, origin and function of the long, slender, 'bacterial forms' observed in the 'altered cells' included in group (ii) above, are uncertain at this stage. The intracellular production of L-forms by Brucella and other species of bacteria grown in cell cultures has been reported by other workers (Hatten and Sulkin, 1966a, b; Egwu and Eveland, 1979), but no attempts were made in this present work to isolate L-forms of mucosalis. The fact that the unidentified 'novel bacterial forms' in some infected cell cultures were shown to stain specifically in immunofluorescence tests with mucosalis antiserum clearly indicates a relationship with this organism (p. 193).

GENERAL DISCUSSION

GENERAL DISCUSSION

In this investigation an attempt has been made to evaluate the growth characteristics of C. sputorum ss mucosalis in different types of cell cultures. There has been no report on the behaviour of this organism in cultured cells and this work was, therefore, primarily intended to obtain an overall picture of mucosalis-cell culture interactions rather than to investigate a limited area in depth. Such an approach was considered as an alternative to animal experiments on PIA largely because of continued lack of success with these procedures. It was hoped that such in vitro studies might indicate features of cellular infection that are of importance and so lead to the development of a successful method of animal experimentation.

Despite extensive investigations by Dr Lawson and his colleagues, many aspects of this disease condition remain to be understood. For example, although mucosalis organisms have been consistently demonstrated within the cytoplasm of adenomatous epithelium and have been isolated in large numbers from affected tissues, exposure of neonatal or post-weaned conventional pigs to mucosalis results in only limited colonisation of the alimentary tract and the disease condition is not produced (Roberts et al., 1980a, b). Exposure of neonatal pigs to mucosalis and infected mucosa obtained from the natural disease, has apparently resulted in the transmission of the disease (Roberts et al., 1977b). However, the experimental conditions were not fully controlled

and as the repeat experiments involving controls have proved to be unsuccessful the method appears to be inadequate for regular transmission of the disease under experimental conditions (Lawson, personal communication). In addition, exposure of gnotobiotic piglets to mucosalis results in colonisation of the alimentary tract with this organism but intracellular bacteria were not demonstrable (Lawson and McCartney, personal communication). In view of these observations and in the absence of any clear cut method by which the condition can be investigated, it seemed that a better understanding of the relationship between the bacteria and cell cultures in vitro might yield information of value in elucidating some of the features that influence the production of the disease in the pig.

There is now considerable evidence to show that several pathogenic microorganisms have the ability to adhere to host cells (Jones, 1977; Arbuthnott and Smyth, 1979) and in some cases it is clear that this is an important factor in the pathogenicity of the relevant organism (e.g. E. coli, V. cholerae, S. agalactiae, etc). In the present work, the attachment of C. sputorum ss mucosalis to a wide range of cell cultures obtained from different species of animals has been demonstrated. Mucosalis organisms show only a 'transitory' attachment to the surfaces of cell cultures and in this respect resemble the adherence demonstrated by V. cholerae to the intestine of the rabbit (Nelson et al., 1976). The adhesive properties of mucosalis organisms appear to be complex and

unlike those of other Gram-negative bacteria in that this organism does not possess any cell surface appendages such as fimbriae or fibrillar antigens which are known to be involved in bacterial adhesion to cell surfaces. Experimental evidence obtained in this study clearly suggests that active motility of mucosalis plays an important role in enhancing bacterial attachment to the surfaces of permissive cell cultures, although the polar flagellum did not appear to act as an adhesive appendage. The importance of bacterial motility in the adhesive process of V. cholerae has been well documented in both in vitro and in vivo systems and non-motile variants of this organism are invariably non-adhesive (Freter and Jones, 1976; Jones and Freter, 1976; Guentzel and Berry, 1978).

Electron micrographs of cell cultures inoculated with mucosalis showed that the bacterium adhered through its cell surfaces, which indicates the possible location of the receptors for bacterial adhesion. The involvement of cell surface components in the adhesive process has been confirmed by the demonstration of selective binding of the ultrasonic disintegrates of mucosalis to different types of cell cultures. In recent years considerable experimental evidence has been accumulated to indicate the importance of bacterial cell surface components in the adhesive process (Smith, 1977; Gibbons, 1980; Ward and Berkeley, 1980).

The 'transitory' nature of the attachment of mucosalis organisms to cells, as well as their absence from the cell surfaces after 24 hr despite 'parasitic growth', requires explanation. A number of factors could be involved. The bacteria may lose their ability to adhere after a period of attachment, as takes place with V. cholerae; bacteria replicating under the conditions of growth in cell culture may lack the features that allow attachment, or possibly some change takes place in the host-cell surface after it has been exposed to bacterial infection. It has also been suggested that phenotypic variation of bacterial cell wall structures can occur under limiting growth conditions as shown by continuous culture techniques (Ellwood and Tempest, 1972; Ellwood, 1975; Hohman and Wilson, 1975), and that microorganisms grown in vitro may be incomplete as regards surface layers or structures, compared with those grown in vivo (Smith, 1977). For example, the surface layers of gonococci grown in 'subcutaneous chambers' in guinea-pigs differ completely from those grown in vitro (Novotny et al., 1975; Arko et al., 1976), and it has been suggested that these morphological differences are related to changes in the antigenic and biological characteristics of these organisms (Ward et al., 1970; Arko et al., 1976; Penn et al., 1976, 1977). Similar changes were reported by Nagy et al., (1977) who found that the production of richly piliated variants of enteropathogenic E. coli 987 (987 P^+) occurred readily in vivo but not in vitro.

It may be, therefore, that mucosalis organisms grown in cell cultures, as in the present study, have an

altered coat which could account for the absence of prolonged attachment of the bacteria to cultured cells. In this context it is worth recalling that mucosalis organisms consistently show phenotypic variation resulting in the recognition of at least three distinct cell surface coats and that 'parasitic' bacteria obtained from an infected 21-day-old BK cell line predominantly contained smooth surfaced mucosalis organisms.

Although the demonstration of the ability of mucosalis organisms to attach to a variety of cell cultures including primary pig kidney cells has indicated the mechanism by which the organism may gain access to the enterocyte, it would be more conclusive if a similar attachment can be demonstrated with isolated intestinal brush-borders of pigs. This feature would obviously more accurately suggest the capacity for intestinal colonization. The use of a single in vitro system as a measure of bacterial adhesion may prove to be misleading and may not represent the in vivo situation. This fact has been clearly demonstrated with V. cholerae in a comprehensive series of experiments conducted by Jones and his colleagues. These workers used three in vitro model systems, namely attachment of V. cholerae to rabbit intestinal brush borders, haemagglutination of human erythrocytes and attachment to slices of rabbit ileal mucosa. They proved that different adhesins were involved in each of these systems. For example, L-fucose was shown to inhibit adhesion of V. cholerae to erythrocytes and isolated brush borders but not adherence to intestinal slices. Moreover, unlike the other two

systems, the attachment of vibrios to ileal mucosa required calcium ions and the presence of D-mannose appeared to inhibit this adhesion process (Freter and Jones, 1976; Jones and Freter, 1976; Jones et al., 1976.

Host-cell surface determinants involved in the attachment of mucosalis to cell cultures are not fully understood but the evidence obtained from a pilot experiment indicated that mannose-like receptors do not appear to be associated with mucosalis attachment. Also bacterial attachment studies involving 'altered cells' induced by mucosalis clearly showed that the organisms failed to attach to these cells due, possibly, to the absence of specific cell surface receptors for the adhesins of mucosalis. Similarly, the inability of CEF, Vero and $LLCMK_2$ cells to attach mucosalis organisms suggests the absence of cell surface receptors. Although significant progress has been made in the last decade or so on the nature of adhesins present on bacterial cell surfaces, similar information on host cell surface determinants appears mostly to be lacking.

Mucosalis has many advantages as a means of investigating host-cell receptors for bacterial adhesion since it both lacks the sophisticated cell surface appendages involved in some other adhesive processes(e.g. pili, fibrillar adhesins, etc) and,as the organism fails to grow in cell-free tissue culture media,in vitro experimental techniques can more easily be manouvered. However, compared with gonococci a great deal of information remains to be obtained on the chemical structure of the cell walls of mucosalis organisms.

The phase of bacterial attachment is invariably followed by phagocytosis of mucosalis organisms, and electron microscopic evidence suggests that the bacteria does not appear to actively penetrate the cell membrane but are engulfed by the host-cell. The engulfment of mucosalis organisms appears to be identical to that observed with N. gonorrhoeae by monkey kidney cells (Waitkins and Flynn, 1973) and Y. pseudotuberculosis by HeLa cells (Bovallius and Nilsson, 1975). In the intracellular location mucosalis was predominantly present within the phagosomes with occasional bacteria lying free in the cytoplasmic substance, usually unbound by the host-cell membranes.

There is an enormous amount of literature on the intracellular growth of pathogenic bacteria in cell cultures. These studies have been mainly restricted to the use of the light microscopy (including immunofluorescence) to demonstrate the intracellular location of bacteria and therefore many of the observations may not be totally valid since many apparently intracellular bacteria may be located on the cell surface as was shown by Swanson (1973), who used amnion cells infected with gonococci. This type of criticism may be applied to many of the previous studies reported in Chapter I.

The intracellular location of organisms is clearly demonstrable in thin-sections of infected cell cultures but electron microscopic studies have been limited to five species of bacteria, namely M. lepraemurium in rat fibroblasts (Brown and Draper, 1970); B. abortus in baby

hamster kidney cells (Hatten et al., 1971); N. gonorrhoeae in monkey kidney cells ($LLCMK_2$ and Vero) and mouse fibroblasts (Waitkins and Flynn, 1973); Y. pseudotuberculosis in HeLa cells (Bovallius and Nilsson, 1975) and B. canis in $LLCMK_2$ cells (Egwu and Eveland, 1979).

In all these cases, the majority of intracellular bacteria were present in cytoplasmic vacuoles, but some were present in the cytoplasmic substance and were generally not enclosed within host-cell membranes.

The intracellular growth characteristics of mucosalis in PK_{pi} and BK cells are of particular interest since infection of these cell lines present distinctive features. Cultures of PK_{pi} cells appear to disintegrate intracellular bacteria rapidly, and intact vibriod organisms were not observed after 24 to 48 hr post-inoculation. The phagosomes, which originally contained mucosalis, were gradually filled with disintegrated 'granular material'. This 'substance' has a close resemblance to the bacterial outer cell wall components obtained after treatment with formaldehyde. It should be emphasized, however, that a strict comparison of these substances cannot be attempted on a morphological basis alone in view of the different techniques employed for their demonstration in the two locations. Nevertheless, the significant aspect of intracellular destruction of mucosalis in PK_{pi} cells has been the production of increasing amounts of 'granular material' in the cytoplasm. Very little of this material is present in the cell cytoplasm at the end of the 2nd day but large amounts of

the same substance appears to be 'produced' in the cell resulting in increased accumulation during the next 2 or 3 days. There was no evidence of further breakdown of this 'substance' and it remained in the same 'granular state' until the destruction of the infected cell.

The identity of the 'granular material' has been further confirmed by immunofluorescence staining of corresponding coverslip preparations which clearly showed specific fluorescence with mucosalis-antiserum. Evidence was also obtained to confirm the particulate nature of the 'granular material' and, in particular, its peripheral location and increased accumulation during the course of mucosalis infection.

The 'granular material' produced in PK_{pi} cells as a result of intracellular penetration by mucosalis organisms does not appear to be restricted to this organism alone, since a similar substance has been observed in rat fibroblasts infected with M. lepraemurium. Brown and Drapper (1970) described this M. lepraemurium-associated material as 'debris', which was clearly recognisable in electron micrographs. They also demonstrated the presence of bacteria as well as 'debris' within the same phagosome, and this these workers took as evidence to prove that this 'debris' was of bacterial origin. The general appearance and location of M. lepraemurium 'debris' within the cell cytoplasm closely resembles that observed in the present study with infected PK_{pi} cells.

In contrast, infected BK cells presented a different picture which indicated a limited destruction of mucosalis organisms, particularly those located in the phagosomes with the production of 'ghost cells' and a total absence of the 'granular material'. In addition, evidence was obtained to suggest that mucosalis is capable of intracellular multiplication in this cell line. There was a gradual increase in the number of intracellular bacteria and thin-sections of infected 21-day-old cultures showed large numbers of morphologically normal intracellular organisms. This was also confirmed by immunofluorescence staining of corresponding preparations which undoubtedly showed enormous numbers of intracellular vibrioid organisms. The presence of several 'peripheral bodies' observed in 21-day-old infected BK cells is intriguing. Although the relationship of these 'bodies' to mucosalis organisms remains to be established, it is postulated that they may represent 'altered forms' of mucosalis. The presence of such structures is not restricted to cell cultures infected with mucosalis since Hatten et al., (1971) observed large numbers of 'altered forms' of B. abortus in 7-day-old infected BHK cells and considered them to be L-forms produced, particularly, under conditions adverse to bacterial survival.

There is little information available about intracellular multiplication of bacteria in cell cultures and in particular about the 'progeny forms', if any, obtained after such intracellular growth. Of the other four bacterial species (B. abortus, M. lepraemurium,

Y. pseudotuberculosis, N. gonorrhoeae) examined by electron microscopy there has been no clear evidence of persistent intracellular growth and Hatten et al., (1971) reported that failure to observe an increase in intracellular B. abortus organisms could be due to the bacteria becoming dormant, destruction by cellular enzymes, antibiotics present in the medium or a combination of these factors. Bovallius and Nilsson (1975) investigated the intracellular growth of Y. pseudotuberculosis in HeLa cells and found that intracellular conditions restricted the growth of this organism to such an extent that attempts to recover bacteria from the infected cultures were unsuccessful after 3 days post-inoculation. Studies on gonococci and M. lepraemurium were limited to the demonstration of organisms in an intracellular location, although, in the latter, apparently healthy looking organisms were clearly demonstrable in electron micrographs even after 6 to 7 days post-inoculation, in addition to the presence of 'bacterial debris' within the cytoplasm. Therefore, in the present investigation, the demonstration of intracellular bacteria in increasing numbers upto 21 days post-inoculation suggests that at least certain types of cell cultures may support or permit the intracellular growth of mucosalis organisms and that this is possibly the first report of persistent multiplication in tissue culture of a non-obligate intracellular bacterial pathogen.

In addition to the demonstration of the organism, 'viable counts' of mucosalis in infected cell cultures

have suggested that intracellular growth of this organism had occurred in PK_{pi} and BK cells. At this stage, it is presumed that the 'parasitic bacteria' present in the supernatant fluids of infected cell cultures are being released into the medium after a phase of intracellular growth. Such an assumption is based on the fact that certain cell cultures failed to support 'parasitic growth' and that this organism is rapidly killed in cell-free tissue culture medium.

The use of a wide range of cell types in the present study provided an opportunity to understand the behaviour of this organism in different cell culture systems. This has resulted in the recognition of certain cell types that were unable to attach mucosalis organisms, support its parasitic growth or show cellular changes induced by this organism. Despite the ability of these bacteria to produce profound cellular changes in a short period, infection did not interfere with initial monolayer formation by cell cultures. In all the cell lines that became infected, but resisted the destructive effects of the organism, a small percentage of cells remained apparently normal and were able to multiply without being reinfected despite the presence of 'parasitic bacteria'. Therefore, it has been possible to eliminate completely not only 'parasitic growth' of mucosalis but also mucosalis-induced altered cells during serial blind passages of certain cell cultures.

In the present investigation, the available experimental evidence suggests that the cytopathic effects

observed in infected cell cultures are associated with the intracellular growth and/or the presence of breakdown products of mucosalis organisms. In infected BK cells, intracellular antigens of mucosalis could be detected upto 55 days post-inoculation by immunofluorescence staining despite serial passage of the infected monolayers. The fact that the 'altered cells' produced by mucosalis behave differently from those of the uninfected parent cells clearly suggests that this organism is capable of inducing functional as well as structural changes in the affected cells. The production of cell fusion by mucosalis organisms both in NDV-infected (after pretreatment with NDV-antiserum) and normal cell cultures is intriguing. Several animal viruses have been known to induce cell fusion in a variety of cell cultures and fusion of cells by inactivated Sendai virus, herpesvirus, and Newcastle disease virus appears to be due to viral envelope components (Smith, 1964, 1977; Hosaka, 1970; Poste, 1972). Fusion factors associated with the envelope of NDV have not yet been separated and identified, and envelope fragments containing these factors have less fusing activity than the intact virus (Poste, 1972). The chemical composition of mucosalis cells is not known and therefore it is of interest that ultrasonic disintegrates (crude, 1 μm or 0.45 μm filtrates) of mucosalis organisms produced extensive cell fusion similar to that observed with intact bacteria.

In retrospect, the present work has been an introduction to the behaviour of mucosalis in cell culture systems and, as such, has thrown some light on the pathogenic potential of this organism with respect to its natural host, the pig. The demonstration of the ability of mucosalis to attach to the surfaces of pig kidney cells, including primary cell cultures, and its subsequent phagocytosis by these cell types points to some of the mechanisms that are likely to be involved in the entry of mucosalis into the intestinal epithelial cells of the pig.

Ultrathin sections prepared from adenomatous epithelium, unlike those of infected cell cultures, clearly show dividing forms of mucosalis and, in general, the bacteria seen in the tissues are less pleomorphic with limited degenerative changes. This difference between the behaviour of the organism in cell lines, as compared with the enterocyte, may be explained in a number of ways. It may be that mucosalis multiplies better within the enterocyte than in tissue culture cells or that the enterocyte has been modified in some way so that its bactericidal mechanisms are suppressed before the entry of the bacteria into the cell. Future work should investigate these avenues possibly using established lines of intestinal cells (e.g. Intestine 407 cell line of Henle and Deinhardt, 1957), employing organ cultures of intestine from gnotobiotic piglets or perhaps using infected diseased tissue in a search for agents capable

of promoting intracellular multiplication.

The intracellular behaviour of mucosalis within the adenomatous epithelium is not fully understood because the absence of a suitable experimental animal system clearly precludes any examination of the dynamics of the infection. This present work at least gives some indication of the time interval which may be involved in the development of the intestinal cell abnormality in the pig although clearly this has to be separated from the postulated epithelial colonization which theoretically must precede it. A feature of the natural disease has been the absence of organisms at the cell surface in parasitized cells and the present observation that modified 'altered' cells in vitro do not attach mucosalis provides an explanation for this phenomenon.

Recently, Lawson et al., (1979) have demonstrated an accumulation of immunoglobulins, particularly IgA, within the infected enterocytes and have postulated that these antibodies might be directed towards intracellular mucosalis. It is possible, therefore, that the bacteria are either being constantly inactivated or that as such IgA antibody is often not lethal to bacteria it is in some way protective to the organism. Possibly such an explanation also gains support from the ease of recovery of mucosalis from such cases in which IgA is present in the intestinal cells.

The growth characteristics of mucosalis in cell culture observed in this work may prove of use in the development of a

more satisfactory rational approach for animal experimentation. For example, the transitory attachment and phagocytosis of mucosalis demand examination of tissues exposed for less than 12 hr post-inoculation. Examination of the ilea of infected animals, the use of perfused intestinal and other tissue slices of pigs might prove useful in the evaluation of the initial interaction of mucosalis with host-cells. The extension of experiments along these lines should clearly indicate whether the cells of exposed neonatal pigs become infected following exposure.

The part played by the host-cell surface receptors in the attachment of mucosalis may be worthy of further investigation. Jones et al., (1976) using rabbit intestinal slices were able to show host-cell surface receptors to V. cholerae and inhibition of bacterial attachment after blocking these receptors. Clearly the role of antibody in blocking the attachment of mucosalis could be critical but the lack of success in achieving intracellular parasitism in colostrum deprived piglets suggests that this mechanism is not the only one operating. The demonstration of genetically determined adherent and non-adherent pigs capable of being colonized by $K88^+$ E. coli indicate the possibility that all pigs might not carry receptors for mucosalis. The widespread distribution of mucosalis antibody in the pig population seems to make this an unlikely explanation (Lawson, personal communication).

In view of the unsuccessful attempts to produce PIA after feeding cultures obtained from bacteriological media, it might be preferable to use mucosalis organisms grown in cell culture systems ('parasitic bacteria') as the bacterial inoculum in future experimental animal infections. Loss of virulence or poor adaptation to intracellular growth are possible reasons for the failure to induce disease with in vitro cultures. However, the failure to reproduce disease using diseased mucosa as inoculum suggests that tissue culture derived bacteria are not really likely to have great success and therefore such a technique should not have any great priority in future experiments.

In conclusion, C. sputorum ss mucosalis is a fascinating organism whose growth characteristics in cell culture systems are complex and intriguing. There appears to be a great deal of scope for further investigations of this unusual 'host-parasite' relationship which may eventually lead to a clearer understanding of the natural disease.

REFERENCES

REFERENCES

ADSERSEN, V. (1932). Cited by Roberts, L. (1978).

AFSHAR, A. (1967). Journal of General Microbiology., 47, 103.

ALDRIDGE, K.E. and COLE, B.C. (1978). Infection and Immunity., 21, 328.

ALKAN, M.L., OFEK, I. and BEACHEY, E.H. (1977). Infection and Immunity., 18, 555.

ALLAN, I. and PEARCE, J.H. (1977). FEMS Microbiology Letters., 1, 211.

ALLAN, I., SPRAGG, S.O. and PEARCE, J.H. (1977). FEMS Microbiology Letters., 2, 79.

ANDERSON, D.R., HOPPS, H.E., BARILE, M.F. and BERNHEIM, B.C. (1965). Journal of Bacteriology., 90, 1387.

ARBUCKLE, J.B.R. (1970). Journal of Medical Microbiology., 3, 333.

ARBUTHNOTT, J.P. and SMYTH, C.J. (1979). In Adhesion of Microorganisms to Surfaces., pp 165-198, Ed. Ellwood, D.C., Melling, J. and Rutter, P.R. Academic Press, London.

ARKO, R.J., BULLARD, J.C. and DUNCAN, W.P. (1976). British Journal of Venereal Diseases., 52, 316.

BARROW, P.A., BROOKER, B.E., FULLER, R. and NEWPORT, M.J. (1980). Journal of Applied Bacteriology., 47, 147.

BAYER, R.C., CHAWAN, C.B. and BIRD, F.H. (1975). Poultry Science., 54, 703.

BERGELAND, M.E., McADARAGH, J.P., WOHLGEMUTH, K. and STOTZ, I. (1975). In 18th Annual Proceedings American Association of Veterinary Laboratory Diagnosticians., p.31.

BIESTER, H.E. and SCHWARTE, L.H. (1931). American Journal of Pathology., 7, 175.

BIESTER, H.E., SCHWARTE, L.H. and EVELETH, D.F. (1939). American Journal of Pathology., 15, 385.

BLOOD, D.C. and HENDERSON, J.A. (1974). 'Veterinary Medicine', 4th Edition, Balliere Tindall, London.

BOATMAN, E., CARTWRIGHT, F. and KENNY, G.E. (1976). Cell Tissue Research., 170, 1.

BOVALLIUS, A. and NILSSON, G. (1975). Canadian Journal of Microbiology., 21, 1997.

BOZEMAN, F.M., HOPPS, H.E., DANAUSKAS, J.X., JACKSON, E.B. and SMADEL, J.E. (1956). Journal of Immunology., 76, 476.

BRAUN, W., GORELICK, A., KRAFT, M. and MEAD, D. (1951). Journal of Infectious Diseases., 89, 286.

BRINTON, C.C. (1965). Transactions of the New York Academy of Sciences., 27, 1003.

BRODEUR, B.R., JOHNSON, W.M., JOHNSON, K.G. and DIENA, B.B. (1977). Infection and Immunity., 15, 560.

BROOKER, B.E. and FULLER, R. (1975). Journal of Ultrastructure Research., 52, 21.

BROOKER, B.E. and FULLER, R. (1977). In. Microbial Ultrastructure., pp.87-100. Ed. Fuller, R. and Lovelock, D.W. Academic Press, New York.

BROWN, C.A. and DRAPER, P. (1970). Journal of Pathology., 102, 21.

BROWNLEE, A. and MOSS, W. (1961). Journal of Pathology and Bacteriology., 82, 513.

BRUNIUS, G. (1980). FEMS Microbiology Letters., 7, 45.

BUCHANAN, T.M., PEARCE, W.A. and CHEN, K.C.S. (1978). In Immunobiology of Neisseria Gonorrhoeae, pp. 242-249. Ed. Brooks, G.F., Gotschlich, E.C., Holmes, K.K., Sawyer, W.D. and Young, E.E. American Society for Microbiology, Washington D.C.

BURGDORFER, W., SEXTON, D.J., GERLOFF, R.K., ANACKER, R.L., PHILIP, R.N. and THOMAS, L.A. (1975). Infection and Immunity., 12, 205.

BURGER, M.M. (1973). Federal proceedings, Federation of the American Society for Experimental Biology., 32, 91.

BUTLER, M. (1969). Nature (London)., 224, 605.

BYRNE, G.I. (1976). Infection and Immunity., 14, 645.

BYRNE, G.I. (1978). Infection and Immunity., 19, 607.

BYRNE, G.I. and MOULDER, J.W. (1978). Infection and Immunity., 19, 598.

CARNEY, JR., F.E. and TAYLOR-ROBINSON, D. (1973). British Journal of Venereal Diseases., 49, 435.

CHERRY, J.D. and TAYLOR-ROBINSON, D. (1970). Applied Microbiology., 19, 658.

COLLIER, A.M. and CLYDE, JR., W.A. (1971). Infection and Immunity., 3, 694.

COLLIER, A.M., CLYDE, JR., W.A. and DENNY, F.W. (1969). Proceedings of the Society for Experimental Biology and Medicine., 123, 790.

CORDES, D.O. and DEWES, H.F. (1971). New Zealand Veterinary Journal., 19, 108.

CRAWFORD, J.G. and FISHEL, C.W. (1959). Journal of Bacteriology., 77,465.

CROHN, B.B. and TURNER, D.A. (1952). Gastroenterology., 20, 350.

CRUICKSHANK, R. (1965). 'Medical Microbiology', 11th Edition, E. and S. Livingstone Ltd., Edinburgh.

DEAS, D.W. (1960). Veterinary Record., 72, 65.

DODD., D.C. (1968). Pathologica Veterinaria., 5, 333.

DOYLE, L.P. (1944). American Journal of Veterinary Research., 5, 3.

DOYLE, L.P. (1948). American Journal of Veterinary Research., 9, 50.

DUBOS, R., SCHAEDLER, R.W., COSTELLO, R. and HOET, P. (1965). Journal of Experimental Medicine., 122, 67.

DUGUID, J.P. (1968). Archivum Immunologiae et Therapiae Experimentalis., 16, 173.

DUGUID, J.P., ANDERSON, E.S. and CAMPBELL, I. (1966). Journal of Pathology and Bacteriology., 92, 107.

DUGUID, J.P., SMITH, I.W., DEMPSTER, G. and EDMUNDS, P.N. (1955). Journal of Pathology and Bacteriology., 70, 335.

DULBECCO, R. and VOGT, M. (1954). Journal of Experimental Medicine., 99, 167.

DULBECCO, R. and FREEMAN, G. (1959). Virology., 8, 396.

EAGLE, H. (1959). Science., 130, 432.

EDWARDS, H.H. (1972). M.Sc. Thesis., University of Edinburgh.

EDWARD, G.A. and FOGH, J. (1960). Journal of Bacteriology., 79, 267.

EGWU, I.N. and Eveland, W.C. (1979). Medical Microbiology and Immunology., 167, 107.

ELLEN, R.P. and GIBBONS, R.J. (1972). Infection and Immunity., 5, 826.

ELLEN, R.P. and GIBBONS, R.J. (1974). Infection and Immunity., 9, 85.

ELLWOOD, D.C. (1975). Proceedings of the Society for General Microbiology., 3, 72.

ELLWOOD, D.C. and TEMPEST, D.W. (1972). Advances in Microbial Physiology., 7, 83.

EMSBO, P. (1951). Nordisk Veterinaermedizin., 3, 1.

EUBANKS, E.R., GUENTZEL, M.N. and BERRY, L.J. (1977). Infection and Immunity., 15, 533.

EVANS, D.G. and EVANS, JR. D.J. (1978). Infection and Immunity., 21, 638.

EVANS, D.G., EVANS, JR. D.J. and TJOA, W. (1977). Infection and Immunity., 18, 330.

EVANS, JR. D.J., EVANS, D.G. and DUPONT, H.L. (1979). Infection and Immunity., 23, 336.

EVANS, D.G., EVANS, JR. D.J., TJOA, W.S. and DUPONT, H.L. (1978). Infection and Immunity., 19, 727.

EVANS, D.G., SILVER, R.P., EVANS, JR. D.J., CHASE, D.G. and GORBACH, S.L. (1975). Infection and Immunity., 12, 656.

EYSSEN, H., SWAELEN, E., KOWSZYK-GINDIFER, Z. and PARAMENTIER, G. (1965). Antonie van Leewenhoek., 31, 241.

FABERGE, A.C. and OLIVER, R.M. (1974). Journal of Microscopie., 20, 241.

FADER, R.C., AVOTS-AVOTINS, A.E. and DAVIS, C.P. (1979). Infection and Immunity., 25, 729.

FIELD, H.I., BUNTAIN, D. and JENNINGS, A.R. (1953). Journal of Comparative Pathology and Therapeutics., 63 153.

FOLLETT, E.A.A. and GORDON, J. (1963). Journal of General Microbiology., 32, 235.

FRASER, K.B. (1976). In Fluorescent Protein Tracing., pp.225-277, Ed. Nairn, R.C. Churchill Livingstone, Edinburgh.

FRASER, G., EDWARDS, H.H., McNULTY, M.S. and RUBEN, J.M.S. (1976). Archives of Virology., 50, 147.

FREEMAN, B.A., KROSS, D. and CIRCO, R. (1961). Journal of Infectious Diseases., 108, 333.

FRETER, R. (1969). Texas Reproductive Biology and Medicine., 27, 299.

FRETER, R., ALLWEISS, B., O'BRIEN, P.C.M. and HALSTEAD, S.A. (1977). Proceedings 13th Joint Conference on Cholera, pp.152-181. Government Printing Office, Washington D.C.

FRETER, R. and JONES, G.W. (1976). Infection and Immunity., 14, 246.

FRIIS, R.R. (1972). Journal of Bacteriology., 110, 706.

FROST, A.J. (1975). Infection and Immunity., 12, 1154.

FUBARA, E.S. and FRETER, R. (1973). Journal of Immunology., 111, 395.

FULLER, R. (1975). Journal of General Microbiology., 87, 245.

FULLER, R., BARROW, P.A. and BROOKER, B.E. (1978). Applied and Environmental Microbiology., 35, 582.

FULLER, R. and BROOKER, B.E. (1980). In Microbial Adhesion to Surfaces., pp.495-507. Ed. Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. Ellis Horwood Ltd., Chichester.

FULLER, R. and TURVEY, A. (1971). Journal of Applied Bacteriology., 34, 617.

GABRIDGE, M.G., GUNDERSON, H., SCHAEFFER, S.L. and BARDEN-STHAL, D.Y. (1978). Infection and Immunity., 21, 333.

GARBUTT, E.W. (1965). International Journal of Leprosy., 33, 578.

GARBUTT, E.W., REES, R.J.W. and BARR, Y.M. (1958). Lancet., ii, 127.

GAVRILESCU, M., LAZAR, M., POROJAN, I., and CIRCIUMARESCU, T. (1966). British Journal of Venereal Disease., 42, 171.

GERBER, D.F. and WATKINS, H.M.S. (1961). Journal of Bacteriology., 82, 815.

GEY, G.O., COFFMAN, W.D. and KUBICECK, M.T. (1952). Cancer Research., 12, 264.

GIANNELLA, R.A., WASHINGTON, O., GEMSKI, P. and FORMAL, S.B. (1973). Journal of Infectious Diseases., 128, 69.

GIBBONS, R.J. (1980). In Microbial adhesion to surfaces., pp.320-388. Ed. Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. Ellis Horwood Ltd., Chichester.

GIBBONS, R.A., JONES, G.W. and SELLWOOD, R. (1975). Journal of General Microbiology., 86, 228.

GIBBONS, R.J., SPINELL, D.M. and SKOBE, Z. (1976). Infection and Immunity., 13, 238.

GIBBONS, R.J. and VAN HOUTE, J. (1971). Infection and Immunity., 3, 567.

GIBBONS, R.J. and VAN HOUTE, J. (1975). Annual Review of Microbiology., 29, 19.

GIBBONS, R.J., VAN HOUTE, J. and LILJEMARK, W.F. (1971). Journal of Dental Research., 51, 424.

GILLIES, R.R. and DUGUID, J.P. (1958). Journal of Hygiene., 56, 303.

GIMENEZ, D.F. (1961). Stain technology., 39, 135.

GORDON, F.B., DRESSLER, H.R., QUAN, A.L., McQUILKIN, W.T. and THOMAS, J.I. (1972). Applied Microbiology., 23 123.

GUBISH, JR. E.R., MACE, JR. M.L., STEINER, S.M. and WILLIAMS, R.P. (1979). Infection and Immunity., 25, 1043.

GUENTZEL, M.N. and BERRY, L.J. (1975). Infection and Immunity., 11, 890.

GUNNARSSON, A., HURVELL, B., JONSSON, L., MARTINSSON, K. and REILAND, S. (1976). Acta Veterinaria Scandinavica., 17, 267.

HARKNESS, A.H. (1948). British Journal of Venereal Disease., 24, 137.

HARRIS, D.L., GLOCK, R.D., CHRISTENSEN, C.R. and KINYON, J.M. (1972). Veterinary Medicine., 67, 61.

HARRIS, D.L. and KINYON, J.M. (1975). Folia Veterinaria Latina., 5, 433.

HATTEN, B.A., HUANG, S.Y., SCHULZE, M.L. and SULKIN, S.E. (1971). Journal of Bacteriology., 108, 535.

HATTEN, B.A. and SULKIN, S.E. (1966a). Journal of Bacteriology., 91, 14.

HATTEN, B.A. and SULKIN, S.E. (1966b). Journal of Bacteriology., 91, 285.

HENLE, G. and DEINHARDT, F. (1957). Journal of Immunology., 79, 54.

HILLMAN, J.D., VAN HOUTE, J. and GIBBONS, R.J. (1970). Archives of Oral Biology., 15, 899.

HOHMANN, A. and WILSON, M.R. (1975). Infection and Immunity., 12, 866.

HOLLAND, J.J. and PICKETT, M.J. (1956). Proceedings of the Society for Experimental Biology and Medicine., 93, 476.

HONDA, E. and YANAGAWA, R. (1975). American Journal of Veterinary Research., 36, 1663.

HOORENS, J. (1962). Cited by Roberts, L. (1978).

HOSAKA, Y. (1970). Journal of General Virology., 8, 43.

HULL, R.N., CHERRY, W.R. and JOHNSON, I.S. (1956). Anatomical Record., 124, 490.

ISSACSON, R.E., NAGY, B. and MOON, H.W. (1977). Journal of Infectious Diseases., 135, 531.

JAMES-HOLMQUEST, A.N., SWANSON, J., BUCHANAN, T.M., WENDE, R.D. and WILLIAMS, R.P. (1974). Infection and Immunity., 9, 897.

JAMES, A.M., KNOX, J.M. and WILLIAMS, R.P. (1976). British Journal of Venereal Disease., 52, 128.

JEPHCOTT, A.E., REYN, A. and BIRCH-ANDERSON, A. (1971). Acta Pathologica et Microbiologica Scandinavica, Section B., 79, 437.

JOHNSON, F.W.A. and HOBSON, D. (1976). Journal of Hygiene., 76, 441.

JOHNSON, AP., TAYLOR-ROBINSON, D. and McGEE, Z.A. (1977). In Gonorrhoea, Epidemiology and Pathogenesis, pp.138-144. Ed. Skinner, F.A., Walker, P.D. and Smith, H. Academic Press, London.

JONES, G.W. (1977). In Microbial interactions., pp.140-168. Ed. Reising, J.L., Chapman and Hall, London.

JONES, G.W. (1980). In Bacterial adherence, pp.219-249. Ed. Beachey, E.H., Chapman and Hall, London.

JONES, G.W., ABRAMS, G.D. and FRETER, R. (1976). Infection and Immunity., 14, 232.

JONES, G.W. and FRETER, R. (1976). Infection and Immunity., 14, 240.

JONES, G.W. and RUTTER, J.M. (1972). Infection and Immunity., 6, 918.

JONES, G.W. and RUTTER, J.M. (1974). Journal of General Microbiology., 84, 135.

JONSSON, L. and MARTINSSON, K. (1976). Acta Veterinaria Scandinavica., 10, 275.

KALIMA, T.V. (1971). Scandinavian Journal of Gastroenterology., 6, 75.

KALIMA, T.V., SALONIEMI, H. and RAHKO, T. (1976). Scandinavian Journal of Gastroenterology., 11, 353.

KANEKO, T. and COLWELL, R.R. (1975). Applied Microbiology., 29, 269.

KEELER, R.F., RITCHIE, A.E., BRYNER, J.H. and ELMORE, J. (1966). Journal of General Microbiology., 43, 439.

KELLOGG, JR. D.S., PEACOCK, JR. W.L., DEACON, W.E., BROWN, L. and PIRKLE, C.I. (1963). Journal of Bacteriology., 85, 1274.

KELLY, W.R. and CAMERON, R.D.A.(1976). In proceedings 53rd Annual Conference of the Australian Veterinary Association, p.146.

KENNY, C.P. and ARIS, B.J. (1969). Canadian Journal of Public Health., 60, 34.

KIHLSTROM, E. (1977). Infection and Immunity., 17, 290.

KIHLSTROM, E. and EDEBO, L. (1976). Infection and Immunity., 14, 851.

KOHLER, W. and PROKOP, O. (1967a). Zeitschrift fur Immunitaestsforschung. 133, 50.

KOHLER, W. and PROKOP, O. (1967b). Zeitschrift fur Immunitaestsforschung. 133, 171.

KORPASSY, B. and TIBOLDI, T. (1957). Cited by Roberts, L. (1978).

KRIEG, N.R. (1976). Bacteriological Reviews., 40, 55.

KUO, C.C. and GRAYSTON, J.T. (1976). Infection and Immunity., 13, 1103.

KUO, C.C., WANG, S.P. and GRAYSTON, J.T. (1972). Journal of Infectious Diseases., 125, 313.

KUO, C.C., WANG, S.P. and GRAYSTON, J.T. (1973). Infection and Immunity., 8, 74.

LABREC, E.H., SCHNEIDER, H., MAGNANI, T.J. and FORMAL, S.B. (1964). Journal of Bacteriology., 88, 1503.

LABREC, E.H., SPRINZ, H., SCHNEIDER, H. and FORMAL, S.B. (1965). In proceedings of the cholera research symposium, pp.262-276. U.S. Public Health Service Publication No. 1328. U.S. Government Printing Office, Washington D.C.

LAI, C., LISTGARTEN, M. and ROSAN, B. (1973). Infection and Immunity., 8, 475.

LAMBDEN, P.R., HECKELS, J.E., JAMES, L.T. and WATT, P.J. (1979). Journal of General Microbiology., 114, 305.

LAMBDEN, P.R., ROBERTSON, J.R. and WATT, P.J. (1980). Journal of Bacteriology., 141, 393.

LAMBDEN, P.R., ROBERTSON, J.N. and WATT, P.J. (1981). Journal of General Microbiology., 124, 109.

LAWSON, G.H.K. and ROWLAND, A.C. (1974). Research in Veterinary Science., 17, 331.

LAWSON, G.H.K., ROWLAND, A.C. and ROBERTS, L. (1975a). Veterinary Record., 97, 308.

LAWSON, G.H.K., ROWLAND, A.C. and ROBERTS, L. (1976). Journal of Medical Microbiology., 9, 163.

LAWSON, G.H.K., ROWLAND, A.C., ROBERTS, L., FRASER, G., and McCARTNEY, E. (1979). Research in Veterinary Science., 27, 46.

LAWSON, G.H.K., ROWLAND, A.C. and WOODING, P. (1975b). Research in Veterinary Science., 18, 121.

LECCE, J.G., KING, M.W. and MOCK, R. (1976). Infection and Immunity., 14, 816.

LEDERBERG, J. and LEDERBERG, E.M. (1952). Journal of Bacteriology., 63, 399.

LEVY, N.J. (1979). Infection and Immunity., 25, 946.

LILJEMARK, W.F. and GIBBONS, R.J. (1972). Infection and Immunity., 6, 852.

LOBO, M.C. and MANDELL, G.L. (1973). Proceedings of the Society for Experimental Biology and Medicine., 142, 1048.

LOUZA, A.C. (1977). Ph.D. Thesis., University of Edinburgh.

LOVE, R.J. and LOVE, D.N. (1977). Veterinary Record., 100, 473.

LOVE, R.J., LOVE, D.N. and EDWARDS, M.J. (1977). Veterinary Record., 100, 65.

LUSSIER, G. (1962). Canadian Veterinary Journal., 3, 267.

MACPHERSON, I.A. and STOKER, M.G.P. (1962). Virology., 16, 147.

MADIN, S.H. and DARBY, N.B. (1958). Proceedings of the Society for Experimental Biology and Medicine., 98, 574.

MANDELL, G.L. (1973). Journal of Clinical Investigation., 52, 1673.

MARDH, P.A. and WESTROM, L. (1976). Infection and Immunity., 13, 661.

MARTINSSON, K. and EKMAN, L. (1974). Svensk Veterinartidning., 26, 824.

MARTINSSON, K., EKMAN, L. and JONSSON, L. (1976). Acta Veterinaria Scandinavica., 17, 233.

MARTINSSON K., HOLMGREN, N., JONSSON, L. and NORDSTROM, G. (1974). Svensk Veterinartidning., 26, 347.

McCOY, E.C., DOYLE, D., WILTBERGER, H., BURDA, K. and WINTER, A.J. (1975). Journal of Bacteriology., 122, 307.

McCOY, E.C., WILTBERGER, H.A. and WINTER, A.J. (1976). Infection and Immunity., 13, 1258.

McGEE, Z.A., JOHNSON, A.P. and TAYLOR-ROBINSON, D. (1976). Infection and Immunity., 13, 608.

McNEISH, A.S., TURNER, P., FLEMING, J. and EVANS, N. (1975). Lancet., ii, 946.

MERRIOT, J., SHOEMAKER, A. and DOWNS, C.M. (1961). Journal of Infectious Diseases, 108, 136.

MEYERS, L.L. and GUINEE, P.A.M. (1976). Infection and Immunity., 13, 1117.

MILES, A.A., MISRA, S.S. and IRWIN, J.O. (1938). Journal of Hygiene, Cambridge, 38, 732.

MOON, H.W., NAGY, B., ISSACSON, R.E. and ØRSKOV, I. (1977). Infection and Immunity., 15, 614.

MORGAN, R.L., ISSACSON, R.E., MOON, H.W., BRINTON, C.C. and TO, C.C. (1978). Infection and Immunity., 22, 771.

MORGAN, S.F., MORTON, H.J. and PARKER, R.C. (1950). Proceedings of the Society for Experimental Biology and Medicine., 73, 1.

MOULDER, J.W., HATCH, T.P., BYRNE, G.I. and KELLOGG, K.R. (1976). Infection and Immunity., 14, 227.

MOYNIHAN, I.W. and GWATKIN, R. (1941). Canadian Journal of Comparative Medicine and Veterinary Science., 5, 167.

MURRAY, R.G.E. (1963). Canadian Journal of Microbiology., 9, 381.

MUSE, K.E., POWELL, D.A. and COLLIER, A.M. (1976). Infection and Immunity., 13, 229.

NAGY, B. (1980). Infection and Immunity., 27, 21.

NAGY, B., MOON, H.W. and ISSACSON, R.E. (1976). Infection and Immunity., 13, 1214.

NAGY, B., MOON, H.W. and ISSACSON, R.E. (1977). Infection and Immunity., 16, 344.

NAIRN, R.C. (1976). Ed. Fluorescent Protein Tracing., 4th Edition, Churchill Livingstone, Edinburgh.

NECOECHEA, R.R., MELGAR, H.C. and GUZMAN de LAS CASAS, M. (1969). Cited by Roberts, L. (1978).

NELSON, E.T., CLEMENTS, J.D. and FINKELSTEIN, R.A. (1976). Infection and Immunity., 14, 527.

NICOLSON, G.L. (1974). International Review of Cytology., 39, 90.

NIELSEN, K. (1967). Acta Veterinaria Scandinavica., 7, 321.

NIELSEN, K. (1971). In Regional Enteritis (Crohns disease)., p.266, Ed. Engel, A. and T. Larsen, Essette Tryck, Stockholm.

NIELSEN, S.V. (1955). Journal of the Americal Veterinary Medical Association., 127, 437.

NOVOTNY, P., SHORT, J.A. and WALKER, P.D. (1975). Journal of Medical Microbiology., 8, 413.

NOWOTARSKA, M. and MULCZYK, M. (1977). Archivum Immunologiae et Therapiae Experimentalis., 25, 7-16.

OFEK, I., MIRELMAN, D. and SHARON, N. (1977). Nature, (London), 265, 623.

ØRSKOV, I., ØRSKOV, F., SMITH, H.W. and SOJKA, W.J. (1975). Acta Pathologica Microbiologica Scandinavica, Section B., 83, 31.

ØRSTAVIK, D., KRAUS, F.W. and HENSHAW, C. (1974). Infection and Immunity., 9, 794.

OTTENSOOSER, F., NAKAMIZO, Y., SATO, M., MIYAMOTO, Y. and TAKIZAWA, K. (1974). Infection and Immunity., 9, 971.

OTTOW, J.C.G. (1975). Annual Review of Microbiology., 29, 79.

OVCINNIKOV, N.M. and DELEKTORSKIJ, V.V. (1971). British Journal of Venereal Diseases., 47, 419.

PATNAIK, B.K. and GHOSH, H.K. (1966). British Journal of Experimental Pathology., 47, 210.

PEAD, P.J. (1979). Journal of Medical Microbiology., 12, 383.

PEARCE, W.A. and BUCHANAN, T.M. (1978). Journal of Clinical Investigation., 61, 931.

PENN, C.W., SEN, D., VEALE, D.R., PARSONS, N.J. and SMITH, H. (1976). Journal of General Microbiology., 97, 35.

PENN, C.W., VEALE, D.R. and SMITH, H. (1977). Journal of General Microbiology., 100, 147.

PIJOAN, C. (1975). British Veterinary Journal, 131, 586.

PILL, A.H. (1971). Veterinary Record., 88, 27.

POSTE, G. (1972). International Review of Cytology., 33, 157.

POSTE, G. and WATERSON, A.P. (1975). In Negative Strand Viruses (Vol 2)., pp.905-922, Ed. Mahy, B.W.J. and Barry, R.D., Academic Press Inc., London.

POTGIETER, L.N.D., FREY, M.L. and ROSS, R.E. (1972). Canadian Journal of Comparative Pathology., 36, 145.

PULLAR, E.M. (1958). Australian Veterinary Journal., 34, 305.

PUNSALANG, A.P. and SAWYER, W.D. (1973). Infection and Immunity., 8, 255.

RAHKO, T. and SALONIEMI, H. (1972a). Nordisk Veterinarmedizin., 24, 132.

RAHKO, T. and SALONIEMI, H. (1972b). Nordisk Veterinaermedizin., 24, 196.

RAHKO, T. and SALONIEMI, H. (1972c). Nordisk Veterinaermedizin., 24, 208.

RAHKO, T. and SALONIEMI, H. (1972d). Suomen elainlaakarilehti., 78, 318. Cited by Roberts L.(1978).

RAHKO, T., SALONIEMI, H. and KALIMA, T.V. (1973). Scandinavian Journal of Gastroenterology., 8, 36.

RAJAN, A., NAIR, M.K. and MARYAMMA, K.I. (1975). Kerala Journal of Veterinary Science., 6, 128.

RAZA, A., SHINEFIELD, H.R., LITZ, C. and MAIBACH, I. (1980). Journal of Infectious Diseases., 141, 463.

REES, R.J.W. and GARBUTT, E.W. (1962). British Journal of Experimental Pathology., XLIII, 221.

REES, R.J.W. and WONG, P.C. (1958). Nature, (London)., 181, 359.

REYNOLDS, E.S. (1963). Journal of Cell Biology., 17, 208.

RHOADES, H.E. (1954). American Journal of Veterinary Research., 15, 630.

RICHARDSON, M. (1959). Journal of Bacteriology., 78, 769.

RICHARDSON, M. and HARKNESS, T.K. (1970). Infection and Immunity., 2, 631.

RICHARDSON, M. and HOLT, J.N. (1962). Journal of Bacteriology., 84, 638.

RICHARDSON, M. and HOLT, J.N. (1964). Journal of Bacteriology., 88, 1163.

RITCHIE, A.E., KEELER, R.F. and BRYNER, J.H. (1966). Journal of General Microbiology., 43, 427.

RIVER, D.A. and DAREKAR, M.R. (1975). Experentia., 31, 662.

ROBERTS, D.S. (1956). Australian Veterinary Journal., 32, 114.

ROBERTS, L. (1978). Ph.D. Thesis., University of Edinburgh.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1977a). Research in Veterinary Science., 23, 257.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1980a). Research in Veterinary Science., 28, 145.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1980b). Research in Veterinary Science., 28, 148.

ROBERTS, L., ROWLAND, A.C. and LAWSON, G.H.K. (1977b). Veterinary Record., 100, 12.

ROBINSON, L.B., WICHELBRASEN, R.H. and RAZINAN, B. (1956). Science., 24, 1147.

RODGER, S.M., CRAVEN, J.A. and WILLIAMS, I. (1975). Australian Veterinary Journal., 51, 536.

ROGERS, H.J. (1979). In Adhesion of Microorganisms to Surfaces; pp.29-55, Ed. Ellwood, D.C., Melling, J. and Rutter, P., Academic Press, London.

ROTA, T.R. and NICHOLS, R.L. (1971). Journal of Infectious Diseases., 124, 419.

ROWLAND, A.C. and LAWSON, G.H.K. (1974). Research in Veterinary Science., 17, 323.

ROWLAND, A.C. and LAWSON, G.H.K. (1975a). Research in Veterinary Science., 18, 263.

ROWLAND, A.C. and LAWSON, G.H.K. (1975b). Veterinary Record., 97, 178.

ROWLAND, A.C., LAWSON, G.H.K. and MAXWELL, A. (1973). Nature, (London)., 243, 417.

ROWLANDS, A.C., LAWSON, G.H.K. and ROBERTS, L. (1976). In Proceedings of the International Pig Veterinary Society., p.N3.

ROWLAND, A.C. and ROWNTREE, P.G.M. (1972). Veterinary Record., 91, 235.

ROWLES, C.R., PARTON, R. and JEYNES, M.H. (1976). In Microbial Ultrastructure., pp.109-115, Ed. Fuller, R. and Lovelock, D.W., Academic Press, London.

RUTTER, J.M. and JONES, G.W. (1973). Nature, (London)., 242, 531.

RUTTER, J.M., JONES, G.W., BROWN, G.T.H., BURROWS, M.R. and LUTHER, P.D. (1976). Infection and Immunity., 13, 667.

SCHAECHTER, M., BOZEMAN, F.M. and SMADEL, J.E. (1957). Virology., 3, 160.

SALIT, I.E. and GOTSCHLICH, E.C. (1977). Journal of Experimental Medicine., 146, 1169.

SALONIEMI, H., OLSONI, E., JANATUINEN, P. and RAHKO, T. (1972). Cited by Roberts, L. (1978).

SANDOK, P.L., JENKIN, H.M., MATTHEWS, H.M. and ROBERTS, M.S. (1978). Infection and Immunity., 19, 421.

SCHAEFER, R.L., KELLER, K.F. and DOYLE, R.J. (1979). Journal of Clinical Microbiology., 10, 669.

SCHERP, H.W. (1971). Science., 173, 1199.

SCHIEFER, H.G., GERHARDT, U., BRUNNER, H. and KRUPE, M. (1974). Journal of Bacteriology., 120, 81.

SCHLESINGER, D. (1975). Ed. Microbiology - 1975, pp.106, 127, 165 and 171. American Society for Microbiology, Washington D.C.

SCHRANK, G.D. and VERWEY, W.F. (1976). Infection and Immunity., 13, 195.

SELLWOOD, R., GIBBONS, R.A., JONES, G.W. and RUTTER, J.M. (1975). Journal of Medical Microbiology., 8, 405.

SHEPARD, C.C. (1955). Proceedings of the Society for Experimental Biology and Medicine., 90, 392.

SHEPARD, C.C. (1957a). Journal of Experimental Medicine., 105, 39.

SHEPARD, C.C. (1957b). Journal of Bacteriology., 73, 722.

SHEPARD, C.C. (1958). Journal of Experimental Medicine., 107, 237.

SHEPARD, C.C. (1959). Journal of Bacteriology., 77, 701.

SHOWACRE, J.L., HOPPS, H.E., DUBUY, H.G. and SMADEL, J.E. (1961). Journal of Immunology., 87, 153.

SILVERBLATT, F.J. (1974). Journal of Experimental Medicine., 140, 1696.

SILVERMAN, D.J. and WISSEMAN, JR. C.L. (1979). Infection and Immunity., 26, 714.

SILVERMAN, D.J., WISSEMAN, JR. C.L. and WADDEL, A. (1980). Infection and Immunity., 29, 778.

SMIBERT, R.M. (1978). Annual Review of Microbiology., 32, 673.

SMITH, H. (1964). Symposium, Society for General Microbiology., 14, 1. Cited by Smith, H. (1977).

SMITH, H. (1976). Symposium, Society for General Microbiology., 26, 299. Cited by Smith, H. (1977).

SMITH, H. (1977). Bacteriological Reviews., 41, 475.

SMITH, H.W. and LINGGOOD, M.A. (1971). Journal of Medical Microbiology., 4, 467.

SMITH, H.W. and LINGGOOD, M.A. (1972). Journal of Medical Microbiology., 5, 243.

SMITH, H. and PEARCE, J.H. (1972). In Microbial Pathogenicity in Man and Animals., pp.25 and 203. Cambridge University Press, Cambridge.

SMYTH, H.F. (1916). Cited by Gerber and Waitkins, 1961.

SOBESLAVSKY, O., PRESCOTT, B. and CHANOCK, R.M. (1968). Journal of Bacteriology., 96, 695.

SOCRANSKY, S.S. (1970). Journal of Dental Research., 49, 203.

SOJKA, W.J. (1971). Veterinary Bulletin., 41, 509.

SOJKA, W.J., WRAY, C. and MORRIS, J.A. (1978). Journal of Medical Microbiology., 11, 493-499.

SPARKES, R.A., PURRIER, B.G.A., WATT, P.J. and ELSTEIN, M. (1977). Cited by Watt, P.J. (1980).

SPEARS, P. and STORZ, J. (1977). Abstract, Annual Meeting of the American Society for Microbiology., D3p.70.

STANBRIDGE, E. (1971). Bacteriological Reviews., 35, 206.

STICE, E. (1955). No. Ref. Cutter Laboratories, Berkeley, California.

SVANBORG-EDEN, C., ERIKSSON, B. and HANSON, L.A. (1977). Infection and Immunity., 18, 767.

SVANBORG-EDEN, C. and HANSSON, H.A. (1978). Infection and Immunity., 21, 229.

SWANSON, J. (1973). Journal of Experimental Medicine., 137, 571.

SWANSON, J., HSU, K.C. and GOTSCHLICH, E.C. (1969). Journal of Experimental Medicine., 130, 1063.

SWANSON, J., KRAUS, S.J. and GOTSCHLICH, E.C. (1971). Journal of Experimental Medicine., 134, 886.

TANNOCK, G.W. and SMITH, J.M.B. (1970). Journal of Comparative Pathology., 80, 359.

TAYLOR, D.J. and ALEXANDER, T.J.L. (1971). British Veterinary Journal., 127, lviii.

TAYLOR-ROBINSON, D., WHYTOCK, S., GREEN, C.J. and CARNEY, JR. F.E. (1974). British Journal of Venereal Disease., 50, 279.

TEBBUTT, G.M., VEALE, D.R., HUTCHINSON, J.G.P. and SMITH, H. (1976). Journal of Medical Microbiology., 9, 263.

VARIAN, A.S. and COOKE, E.M. (1980). Journal of Medical Microbiology., 13, 111.

VERON, M. and CHATELAIN, R. (1973). International Journal of Systematic Bacteriology., 23, 122.

WAITKINS, S.A. and FLYNN, J. (1973). Journal of Medical Mircobiology., 6, 399.

WALKER, P.D. and NAGY, L.K. (1980). In Microbial Adhesion to Surfaces., pp.473-494. Ed.Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. Ellis Horwood Ltd., Chichester.

WALLACE, J.H., ELECK, S.D. and HANKS, J.H. (1958). Proceedings of the Society for Experimental Biology., 97, 101.

WARD, J.B. and BERKELEY, R.C.W. (1980). In Microbial Adhesion to Surfaces., pp.47-66. Ed. Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B., Ellis Horwood Ltd., Chichester.

WARD, M.E. and WATT, P.J. (1972). Journal of Infectious Diseases., 126, 601.

WARD, M.E. and WATT, P.T. (1975). In Genital Infections and their Complications., pp.229-241. Ed. Danielsson, D., Juhlin, L. and Mardh, P.A. Almqvist and Wiksell, Stockholm.

WARD, M.E. and WATT, P.J. (1977). In Gonorrhoeae - Epidemiology and Pathogenesis., pp.83-95. Ed. Skinner, F.A., Walker, P.D. and Smith, H., Academic Press, London.

WARD, M.E., WATT, P.J. and GLYNN, A.S. (1970). Nature, (London)., 227, 382.

WARD, M.E., WATT, P.J. and ROBERTSON, J.N. (1974). Journal of Infectious Diseases., 129, 650.

WARNER, S.D. (1965). Cited by Roberts, L. (1978).

WATT, P.J. (1980). In Microbial Adhesion to Surfaces., pp.445-471. Ed. Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. Ellis Horwood Ltd., Chichester.

WEISS, E. and DRESSLER, H.R. (1960). Proceedings of the Society for Experimental Biology and Medicine., 103, 691.

WERNER, H.J. (1963). American Journal of Veterinary Research., 24, 881.

WERNER, H.J., LEVY, H. and SPURLOCK, B.O. (1961). American Journal of Veterinary Research., 22, 1121.

WESTENDORP, J.F. (1965). Cited by Roberts, L. (1978).

WILLIAMS, P.H., EVANS, N., TURNER, P., GEORGE, R.H. and McNEISH, A.S. (1977). Lancet., 1, 1151.

WILSON, M.H. and COLLIER, A.M. (1976). Journal of Bacteriology., 125, 332.

WILSON, M.R. and HOHMANN, A.W. (1974). Infection and Immunity., 10, 776.

WINTER, A.J., McCOY, E.C., FULLMER, C.S., BURDA, K. and BIER, P.J. (1978). Infection and Immunity., 22, 963.

WISSEMAN, JR.C.L., WADDELL, A.D. and SILVERMAN, D.J. (1976). Infection and Immunity., 13, 1749.

WOODE, G.N., BRIDGER, J., HALL, G.A., JONES, J.M. and JACKSON G. (1976). Journal of Medical Microbiology., 9, 203.

YOKOMIZO, Y. and SHIMIZU, T. (1979). Research in Veterinary Science., 27, 15.

ZUCKER-FRANKLIN, D., DAVIDSON, M. and THOMAS, L. (1966). Journal of Experimental Medicine., 124, 521.